REMSEN

P.O. Box 1450
Alexandria, VA 22313-1450
If Undeliverable Return in Ten Days

AN EQUAL OPPORTUNITY EMP

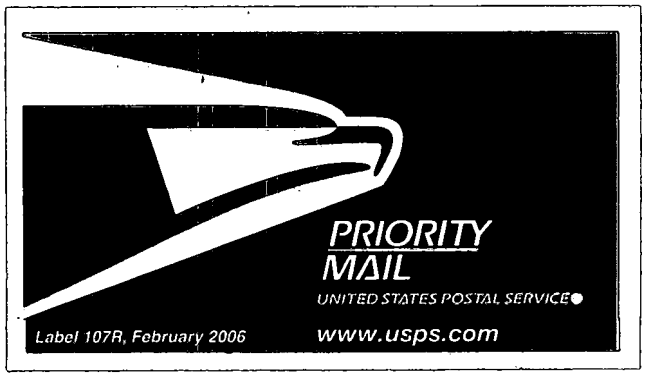
OFFICIAL BUSINESS
PENALTY FOR PRIVATE USE, $300

UNITED STATES POSTAGE
$ 05.75⁰
02 1M
0004244939
SEP 25 2009
MAILED FROM ZIPCODE 22314

NIXIE 3327 1 03 10/27/09

RETURN TO SENDER
NOT DELIVERABLE AS ADDRESSED
UNABLE TO FORWARD
RETURN TO SENDER

RECEIVED
OCT 29 2009
USPTO MAIL CENTER



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/589,956	09/20/2007	Edward H. Cohen	C007-7022US0	2118

56679 7590 09/25/2009

GOSZ AND PARTNERS LLP
ONE STATE STREET
BOSTON, MA 02109

EXAMINER	
HADDAD, MAHER M	
ART UNIT	PAPER NUMBER
1644	

MAIL DATE	DELIVERY MODE
09/25/2009	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

imaging purposes in diagnosis [see, for example, Colcher *et al.* (1988), Schlom *et al.*, (1991)], and in therapy by targeting specific tissues or cells and by delivering toxins or radioactivity to predetermined locations [see, for example, Chaudary *et al.* (1988), and Schlom (1991)].

Furthermore, in view of the similarity in structure of antibodies belonging to the different classes and isotypes, the structure of a particular antibody could be manipulated to generate new functions or to endow it with a desired reactivity. For example, it may be possible, through the construction of chimeric IgE molecules of pre-selected specificity, to harness the cytotoxic potential of the IgE-isotypic response mediated by the low-affinity receptor for IgE and effect the killing of tumor cells and parasites by inflammatory cells (Helm, 1989).

Another important application is the use of antibodies as enzymes. It was proposed (Jencks, 1969) that catalytic antibodies could be generated by the use of transition-state analogs as immunogens, and a number of such antibodies have been produced [see, for example, Lerner and Tramontano (1988), Schultz (1988) and Benkovic (1992)]. This opens the possibility of generating enzymes for chemical reactions for which no natural catalysts exist.

Monoclonal antibodies are still more easily obtained from nonhuman sources, usually rodent, and the use of those antibodies in human subjects will be hindered by the patients' immune system. Since the reduction of the immunogenicity in humans of xenogeneic antibodies will make those molecules more efficacious reagents in therapy and diagnosis, various procedures for "humanizing" nonhuman antibodies are being developed.

Structural considerations in the "humanization" of antibodies

Ideally, "humanization" should result in an antibody that is nonimmunogenic, with complete retention of the antigen-binding properties of the original molecule. However, in order to retain all the antigen-binding properties of the original antibody, the structure of its combining-site has to be faithfully reproduced in the "humanized" version. This could be achieved by transplanting the combining site of the nonhuman antibody onto a human framework, either (a) by grafting the entire nonhuman variable domains onto human constant regions (Morrison *et al.*, 1984; Morrison and Oi, 1988) (which preserves the ligand-binding properties, but which also retains the immunogenicity of the nonhuman variable domains); (b) by grafting only the nonhuman CDRs onto human framework and constant regions (Jones *et al.*, 1986; Verhoeyen *et al.*, 1988); or (c) by transplanting the entire nonhuman variable domains (to preserve ligand-binding properties) but also "cloaking" them with a human-like surface through judicious replacement of exposed residues (to reduce antigenicity) (Padlan, 1991).

(i) *"Humanization" by CDR-grafting.* "Humanization" by transplanting only the CDRs (Jones *et al.*, 1986; Verhoeyen *et al.*, 1988) should result in almost complete elimination of immunogenicity (except if allotypic or idiotypic differences exist). However, it is found that in order to recover the ligand-binding properties of the original molecule, some framework residues from the original antibody, those which influence the structure of the combining site, also need to be preserved [see, for example, Riechmann *et al.* (1988), and Queen *et al.* (1989)].

In theory, all the framework residues which could influence the structure of the combining site should be kept. These include those which are in contact with the CDRs, since they provide the primary support for the combining site structure, and those which are involved in the V_L–V_H contact, since they influence the relative disposition of the CDRs. It may be necessary to keep also those framework residues which are buried in the domain interior, since they could influence the overall domain structure and, thereby, the structure of the combining site. Further, if the interaction with antigen involves "induced fit", the structural elements which permit the conformational change(s) must also be preserved in the "humanized" molecule.

The important framework residues could be identified if the three-dimensional structure of the antibody is known, preferably in a complex with antigen. In the absence of three-dimensional structure, the identification of the important framework residues could be attempted by other means, for example, by modeling the combining site structure (e.g. Queen *et al.*, 1989; Carter *et al.*, 1992), or by studying the effect of framework mutations on the ligand-binding properties of the molecule (e.g. Tempest *et al.*, 1991; Foote and Winter, 1992). Useful hints can be obtained from an examination of the known antibody structures.

The framework residues, which probably play a role in maintaining the combining site structure are presented in Tables 18–21 for the murine antibodies of known structure. The framework residues in the V_L domains, the side chains of which contact CDR residues, are listed in Table 18; those in the V_H are listed in Table 19. The framework residues, which contact framework residues in the opposite domain and which influence the quaternary structure of the Fv, have already been identified (Table 8). The buried, inward-pointing, framework residues in the V_L, i.e. those which are located in the domain interior, are listed in Table 20; those in the V_H are listed in Table 21. These results are collected in Table 22 for V_L and in Table 23 for V_H.

It is seen that there are many framework residues that contact the CDRs, that contact the opposite domain and that are found in the domain interior. These framework residues, which could influence the structure of the combining site and thus the antigen-binding characteristics of an antibody, are different from antibody to antibody, although many are common to all.

If one of the murine antibodies listed in the tables were to be "humanized" by CDR-grafting with the view to preserving their ligand-binding properties, it would probably be wise to retain all of its framework residues that are listed in Tables 22 and 23. At first glance, it would appear that there would be too many nonhuman

Table 18. V_L framework residues that contact CDR residues in murine Fabs of known three-dimensional structure

Position	Antibody														
	J539	McPC603	HyHEL-10	HyHEL-5	R19.9	4-4-20	BV04-01	36-71	B13I2	D1.3	Ysi9-1	AN02	17/9	8F5	NC41
1	E(2)	D(5)	D(10)	D(3)	D(5)	D(8)		D(4)	D(5)	D(11)	D(5)	Q(5)	D(1)	D(5)	D(5)
2	I(11)	I(15)	I(18)	I(18)	I(20)	V(12)	V(9)	I(21)	V(7)	I(12)	I(25)	I(17)	I(15)	I(13)	I(16)
3		V(3)	V(2)	V(3)	Q(20)	V(2)		Q(2)	L(1)		Q(2)	V(2)	V(2)	V(1)	V(3)
4	L(13)	M(11)	L(12)	L(17)	M(14)	M(18)	M(12)	M(9)	M(6)	M(12)	M(9)	L(14)	M(11)	M(7)	M(8)
5		T(3)	T(1)		T(1)	T(3)	T(2)			T(1)	T(1)	T(2)	T(4)	T(4)	T(2)
7					T(3)				T(1)						
22														T(4)	
23	C(3)	C(3)	C(4)	C(4)	C(3)	C(3)	C(2)	C(3)	C(3)	C(1)	C(3)	C(5)	C(5)	C(3)	
35	W(8)	W(6)	W(7)	W(8)	W(8)	W(5)	W(6)	W(10)	W(7)	W(10)	W(9)	W(6)	W(7)	W(7)	W(3)
36	Y(17)	Y(21)	Y(12)	Y(10)	Y(19)	Y(13)	Y(10)	Y(19)	Y(14)	Y(18)	Y(8)	Y(17)	Y(21)	Y(10)	Y(11)
45					K(5)	K(5)									
46	P(8)	L(9)	L(4)	R(21)	L(8)	V(15)	L(12)	L(8)	L(12)	L(9)	L(7)	L(9)	V(5)	L(14)	L(8)
48	I(10)	I(11)	I(11)	I(12)	I(11)	I(9)	I(10)	I(13)	I(10)	V(9)	I(13)	I(13)	I(9)	I(11)	I(2)
49	Y(36)	Y(33)	K(17)	Y(18)	Y(31)	Y(29)	Y(31)	Y(30)	Y(28)	Y(29)	Y(32)	Y(27)	Y(37)	Y(34)	Y(14)
58	V(8)	V(6)	I(6)	V(11)	V(7)	V(9)	V(12)	V(4)	V(8)	V(6)	V(9)	V(9)	V(8)	V(10)	V(4)
60		D(1)				D(2)			D(1)			V(1)	D(2)	D(4)	D(2)
62				F(1)	F(1)	F(1)	F(1)	F(1)				F(1)			
63									S(1)					T(1)	
67		S(3)	S(2)							S(1)			S(2)	S(4)	
69	T(5)	T(9)	T(8)	T(2)	T(8)	T(14)	T(7)	T(8)	T(4)	T(9)	T(9)	T(10)	T(4)	T(6)	
70		D(2)			D(3)		D(2)	D(6)	D(6)		D(9)		D(1)		
71	Y(14)	F(23)	F(23)	Y(23)	Y(21)	F(17)	F(28)	Y(21)	F(21)	Y(25)	Y(25)	Y(12)	F(16)	F(14)	Y(13)
88	C(4)	C(3)	C(3)	C(2)	C(3)	C(3)	C(2)	C(2)	C(2)	C(5)	C(4)	C(4)	C(6)	C(2)	C(1)
98	F(11)	F(15)	F(13)	F(7)	F(14)	F(12)	F(14)	F(12)	F(17)	F(17)	F(14)	F(17)	F(10)	F(16)	F(6)

The number in parentheses after each residue name corresponds to the number of atomic contacts in which the amino acid is involved. See footnote to Table 4 for the definition of contacts. The liganded forms of McPC603, BV04-01, B13I2 and 17/9 (PDB Entry: 1HIM, first Fab in the entry) were used in the computations.

Table 19. V_H framework residues that contact CDR residues in murine Fabs of known three-dimensional structure

Position	Antibody J539	McPC603	HyHEL-10	HyHEL-5	R19.9	4-4-20	BV04-01	36-71	B13I2	D1.3	VST9-1	AN02	17/9	8F5	NC41
1							E(3)	E(3)			E(2)				
2	V(11)	V(3)	V(8)		V(3)			V(7)	V(13)	V(12)	V(3)		V(11)	V(10)	I(6)
4	L(3)	L(6)	L(5)	L(2)	L(1)	L(3)		L(3)	L(3)	L(4)	L(7)	L(5)	L(4)	L(7)	L(2)
22															C(2)
24		T(2)	V(6)			A(1)	A(1)				T(1)	V(5)			A(1)
27	F(3)	F(2)		Y(14)	Y(7)	F(26)	F(2)	Y(4)	F(2)	F(4)	F(8)	Y(16)	F(8)	F(15)	Y(4)
28	D(9)	T(6)		T(3)	T(4)	T(6)	S(1)	T(2)	T(7)		T(8)	S(1)	S(3)		
29	F(4)	F(4)		F(10)	F(6)	F(13)	F(4)	F(6)	F(5)	L(1)	F(4)	I(7)	F(2)	I(5)	F(6)
30	S(2)	S(1)	T(4)	S(3)	T(9)	S(9)	N(1)	T(4)	S(1)	T(2)	T(10)	T(2)	S(1)	K(1)	T(1)
36	W(4)	W(3)	W(6)	W(5)	W(6)	W(7)	W(4)	W(8)	W(4)	W(6)	W(4)	W(3)	W(3)	W(8)	
37		V(1)			V(1)		V(1)		V(4)	V(1)		I(4)	V(2)		
38	R(1)	R(2)	R(4)	K(2)	K(2)	R(4)	R(1)	K(2)	R(1)			R(1)			K(4)
40				R(1)											
46	E(3)	E(4)	E(1)	E(27)	E(7)	E(4)	E(2)	E(9)		E(1)		E(2)		E(4)	E(7)
47	W(34)	W(46)	Y(22)	W(22)	W(36)	W(31)	W(34)	W(36)	W(25)	W(36)	W(28)	W(37)	W(42)	W(37)	W(19)
48	I(1)	I(1)	M(6)	I(12)	I(8)	V(1)	V(4)	I(9)	V(2)	L(1)	L(1)	M(3)		I(1)	M(10)
49		A(4)				A(3)	A(5)		A(3)				A(4)		
66	K(2)	K(3)	R(16)	K(1)	K(2)	R(8)	R(8)	K(1)	R(5)	R(3)	R(2)	R(3)	R(8)	K(3)	
67	F(6)	F(10)	I(9)	A(1)	T(7)	F(13)	F(5)	T(8)	F(10)	L(10)	F(9)	I(10)	F(6)	A(5)	F(9)
68		I(1)				T(11)					T(1)	S(1)	T(3)		
69	I(9)	V(7)	I(12)	F(16)	L(12)	I(25)	I(19)	L(9)	I(12)	I(9)	I(9)	I(10)	I(19)	I(12)	F(16)
71	R(27)	R(28)	R(7)	A(4)	V(2)	R(13)	R(28)	V(7)	R(21)	K(9)	R(23)	R(23)	R(21)	V(4)	L(6)
73	N(3)	T(3)			R(3)	D(3)	D(1)	K(2)	N(1)		N(7)		N(1)	T(1)	
78	L(5)	L(7)	Y(15)	A(1)	A(1)	V(2)	L(3)	A(1)	L(5)	V(4)	L(3)	F(11)	L(6)	A(2)	A(2)
80						L(1)									
82			L(2)							M(1)					
82a				N(1)	R(2)							K(1)			
86						D(2)									
92	C(1)	C(1)	C(1)	C(2)	C(1)			C(2)		C(2)		C(1)	C(1)	C(1)	C(1)
93	A(5)	A(6)	A(1)	L(6)	A(4)	T(6)	V(8)	A(2)	T(6)	A(5)	T(3)	A(2)	A(5)	D(7)	A(1)
94	R(39)	R(26)	N(18)	H(9)	R(35)		R(14)	R(29)	R(27)	R(34)	R(36)	R(39)	R(36)		R(16)
103	W(13)	W(14)	W(15)	W(3)	W(16)	W(7)	W(10)	W(14)	W(7)	W(7)	W(2)	W(12)	W(11)	W(7)	W(3)

See footnote to Table 18.

residues to keep; however, searching through the tabulation of immunoglobulin sequences (Kabat *et al.*, 1991), one finds that there are human variable domain sequences which have most of the framework residues that need to be preserved. For example, the "humanization" of the murine antibody, HyHEL-5, would require keeping nine murine framework residues in the V_L, using the human V_κ sequence, BI (or REI and a few others), as template, and 13 framework residues in the V_H using the human V_H sequence, AND (or 21/28'CL, and a few others). On the other hand, the "humanization" of the murine antibody, B13I2, would require the retention of only three murine framework residues in the V_L, using the human V_κ sequence, CUM (or NIM), as template, and only two framework residues in the V_H, using the human V_H sequence, M72 (or M74 and a few others). It is possible that there exist other human sequences that are even more similar to these murine domains, that are known but were not included in the compilation of Kabat *et al.* (1991).

The results presented in Tables 22 and 23 could be used in the design of a "humanization" protocol for an antibody of unknown structure. If a high degree of sequence similarity to an antibody of known three-dimensional structure exists, the results for the latter could be used. If no obvious sequence similarity exists, the results which are common to all the antibodies analysed could be used as a first guess and the protocol later refined, if needed, by site-directed mutagenesis studies.

It is seen from Tables 22 and 23 that many of the important framework residues flank the CDRs. Among these flanking positions are most of the framework residues that are involved in the contact with the opposite domain (Table 8) and many of those which are in contact with the CDRs (Tables 18 and 19). Moreover, all of the framework residues which have been observed to participate in the binding to antigen (Amit *et al.*, 1986; Sheriff *et al.*, 1987; Padlan *et al.*, 1989; Bentley *et al.*, 1990, Fischmann *et al.*, 1991; Tulip *et al.*, 1992*a*), are in these flanking regions. These results suggest that, if during "humanization", not just the CDRs are transplanted, but also some of the residues immediately adjacent to the CDRs, there would be a better chance of retaining the ligand-binding properties of the original antibody. The likelihood will be even greater if the first few amino acids in the N-termini of both chains are transplanted also, since some of them are found to be in contact with CDRs. In fact, the N-termini are contiguous with the CDR surface and are in a position to be involved in ligand binding (Fig. 4). It should be noted that the N-termini of the light and heavy chains are not equivalent in this regard, with the N-terminus of the light chain being more involved in the contact with the CDRs (this finding may have important implications for the properties of single-chain antibodies where either the end of the heavy chain component is linked to the beginning of the light chain (Huston *et al.*, 1988), or *vice versa* (Bird *et al.*, 1988), especially when residues in the N-terminus are excised).

(ii) *"Humanization" by replacing surface residues to make an antibody more "human-like"*. It may be possible to reduce the antigenicity of a nonhuman Fv, while preserving its antigen-binding properties, by simply replacing those exposed residues in its framework regions which differ from those usually found in human antibodies (Padlan, 1991). This would "humanize" the surface of the xenogeneic antibody while retaining the interior and contacting residues which influence its antigen-binding characteristics. The judicious replacement of exterior residues should have little, or no, effect on the interior of the domains, or on the interdomain contacts.

The solvent accessibility patterns of the Fv of Kol (PDB Entry: 2FB4) and of J539 (PDB Entry: 2FBJ) have been analysed (Padlan, 1991) and are reproduced here. The fractional accessibility values for the framework residues in the Kol and J539 V_L are compared in Table 24 and those for the framework residues in the Kol and J539 V_H are presented in Table 25. Examination of these tables reveals a very close similarity in the exposure patterns of the Fvs of Kol (a human IgG1,λ) and J539 (a murine IgA,κ). Indeed, in almost all the positions in which the two patterns differ significantly, one or both antibodies have glycine. Here, glycine is designated as being completely exposed if its α-carbon is accessible to the solvent probe and completely buried otherwise (see footnote to Table 16), so that the slightest difference in structure could result in a different exposure designation for this amino acid.

The very close similarity of the exposure patterns for the variable domains of Kol and J539 points to the close correspondence of the tertiary structures of the homologous domains and of the dispositions of the individual residues. This is particularly remarkable since (a) these antibodies are from different species, (b) their light chains are of different types (J539 has a κ light chain, while Kol has a λ light chain), (c) half of their CDRs, specifically CDR1-L, CDR3-L and CDR3-H, have very different lengths and backbone conformations (Table 8, Fig. 5), and (d) Kol and J539 have only 44 identical residues out of the 79 corresponding positions in the V_L framework and 60 out of the 87 in the V_H framework. An even closer similarity in overall structure and in the exposure patterns can be expected for two molecules that are more similar in sequence than this pair. These results suggest that the solvent exposure of a residue can be more easily predicted than perhaps its involvement in the maintenance of the combining site structure.

The procedure that was proposed (Padlan, 1991) for reducing the antigenicity of a xenogeneic variable domain, while preserving its ligand-binding properties, would replace only the exposed framework residues which differ from those of the host with the corresponding residues in the most similar host sequence. Thus, the framework residues, which are at least partly exposed in the corresponding domains of Kol or J539 (those with pB, mE, or Ex designations in Tables 24 and 25), would be replaced, while the framework residues, corresponding to those which in Kol and J539 are completely or mostly buried (mB and Bu designations in Tables 24 and

Table 20. Inward-pointing, buried framework residues in the V_L of murine Fabs of known three-dimensional structure

Position	Antibody														
	J539	McPC603	HyHEL-10	HyHEL-5	R19.9	4-4-20	BV04-01	36-71	B13I2	D1.3	Yst9-1	AN02	17/9	8F5	NC41
2	I	I	I	I	I	V	V	I	V	I	I	I	I	I	I
4	L	M	L	L	M	M	M	M	M	M	M	L	M	M	M
6	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
11	T	L	L	M	L	L	L	L	L	L	L	M	L	L	M
13	A	V	V	A	A	V	V	A	V	A	A	A	V	V	T
19	V	V	V	V	V	A	A	V	A	V	V	V	V	V	V
21	I	M	L	M	I	I	I	I	I	I	I	M	M	M	I
23	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
35	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
37	Q	Q	Q	Q	Q	L	L	Q	L	Q	Q	Q	Q	Q	Q
47	W	L	L	W	L	L	L	L	L	L	L	L	L	L	L
48	I	I	I	I	I	I	I	I	I	V	I	I	I	I	I
58	V	V	I	V	V	V	V	V	V	V	V	V	V	V	V
61	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
62	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
71	Y	F	F	Y	Y	F	F	Y	F	Y	Y	Y	F	F	Y
73	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
75	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
78	M	V	V	M	L	V	V	L	V	L	L	M	V	V	V
82	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
83										F					
84	A	A		A	A			A			A	A	A	A	A
86	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
88	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
102	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
104	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
106	L	I	I						I		I	L		L	I

An inward-pointing residue is designated as buried if at least 50% of its side chain is inaccessible to solvent. Solvent accessibilities were computed as described previously (Padlan, 1990*b*); residue exposure is defined in the context of an isolated domain. See footnote to Table 16 for the definition of solvent accessibility. The coordinates used were those considered in Table 18.

Table 21. Inward-pointing, buried framework residues in the V_H of murine Fabs of known three-dimensional structure

Position	Antibody J539	McPC603	HyHEL-10	HyHEL-5	R19.9	4-4-20	BV04-01	36-71	B13I2	D1.3	Yst9-1	AN02	17/9	8F5	NC41
2	V	V	V		V			V	V	V	V	V	V	V	I
4	L	L	L	L	L	L	P	L	L	L	L	L	L	L	L
6	E	E	E	Q	Q	E	E	Q	E	E	E	E	E	Q	Q
12	V	V	V	M	V	V	V	V	V	V	V	V	V	V	K
18	L	L	L	V	V	M	L	V	L	L	L	Q	L	V	V
20	L	L	L	I	M	L	L	M	L	I	L	L	L	L	I
22	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
24	A	T	V	A	A	A	A	A	A	V	T	V	A	T	A
27	F	F		Y	Y	F		Y	F	F	F	Y	F	F	Y
29	F	F	I	F	F	F	F	F	F	L	F	I	F	I	F
36	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
38	R	R	R	K	K	R	R	K	R	R	R	R	R	K	K
40						S							T	R	
46			E	E								E			
48	I	I	M	I	I	V	V	I	V	L	L	M	V	I	M
49		A				A	A		A				A		
66		R	R			R	R		R	R	R	R	R		R
67	F	F	I	A	T	F	F	T	F	L	F	I	F	A	F
69	I	V	I	F	L	I	I	L	I	I	I	I	I	I	F
71	R	R	R	A	V	R	R	V	R	K	R	R	R	V	L
73							D				N		N		
76						S									
78	L	L	Y	A	A	V	L	A	L	V	L	F	L	A	A
80	L	L	L	M	M	L	L	M	L	L	L	L	L	L	L
82	M	M	L	L	L	M	M	L	M	M	M	L	M	L	I
82c	V	L	V	L	L	L	L	L	L	L	L	V	L	L	L
86	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
88	A	A	A		A		A	A	A	A	A	A	A	A	A
90	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	F
92	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
94	R	R	N	H	R		R	R	R	R	R	R	R		R
107	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
109	V	V	V	L	L	V	V	L	L	L	V	V	V	V	L
111	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V

See footnote to Table 20.

25), would be retained. With this procedure also, the number of framework residues in xenogeneic domains, that would be needed to be replaced by human residues, was found to be not very large in the cases examined (Padlan, 1991).

Structural aspects which relate to catalysis by antibodies

Antibodies can be elicited against essentially any structure (if presented properly) so that it may be possible to generate catalytic antibodies of any desired specificity. However, antibodies capable of high enzymatic rates may not be so readily obtainable.

It is difficult to compare the interaction between antibody and antigen and that between enzyme and substrate. While we can analyse the interatomic contacts in an antibody–antigen complex (in the crystal), a comparable study cannot be performed on an enzyme–substrate complex, which is usually of fleeting existence. However, a comparison could be attempted using the more stable enzyme–inhibitor complexes, of which a number have been elucidated by X-ray diffraction.

The interactions of various enzymes with protein and peptide inhibitors are presented in Table 26. By and large, the interactions shown in Table 26 are comparable to those presented in Table 14 between antibodies and their specific ligands, except in one respect: more main chain atoms are found to be involved in the enzyme–inhibitor interactions than in the antibody–ligand complexes. This is especially noticeable in the case of hydrogen-bond formation (see also Janin and Chothia, 1990). In the three anti-lysozyme–lysozyme complexes, on average only 15% of the atomic contacts involve main chain atoms, whereas in the enzyme–protein inhibitor complexes the main-chain involvement is almost three times as great; in regard to the hydrogen bonds, in the anti-lysozyme–lysozyme complexes, only about 7% involve main chain atoms, whereas in the enzyme–protein inhibitor complexes, almost one-half of the hydrogen bonds involve main chain atoms. The number of interatomic contacts between antibody and peptide ligand (Table 14) and between enzyme and peptide inhibitor (Table 26) are roughly the same, but here again there are more hydrogen bonds involving main chain atoms in the enzyme–peptide inhibitor than in antibody–peptide complexes.

The polypeptide backbone is more restricted in its movements than are side chains. Furthermore, hydrogen bonds involving main chain atoms are more "directed" and will be stronger (Jeffrey and Saenger, 1991). Compared to antibody combining sites, the active sites of enzymes are more rigid, being constructed with main-chain and side-chain components that are held in place by strong interactions with surrounding structural elements. A ligand would therefore be interacting with a much less deformable site in an enzyme than in an antibody.

The plasticity of the combining site could have serious implications for the enzymatic activity of catalytic antibodies. A flexible active site cannot produce as much "strain" (Jencks, 1969) on the substrate as could a rigid site and this may result in the diminution of enzymatic efficiency. The observation that most of the binding to antigen involves side chain atoms is perhaps an omen that antibodies with catalytic efficiencies comparable to those of enzymes will be a rarity.

CONCLUDING REMARKS

Antibodies are remarkable molecules and serve as paradigms for macromolecular recognition. They have a binding site that is designed to accommodate ligands of variable structure (the combining site) and sites that bind to ligands of constant structure (e.g. the C1q binding site). The available crystallographic data allow us to assess only the first of these sites (the combining site), but nature's use of structure to achieve a particular function is already evident from this example.

The basic element used in the construction of an antibody molecule is the anti-parallel β-pleated sheet, which is strong because the hydrogen-bonding pattern involves not just one stretch of polypeptide chain, as in an α-helix, but several in a synergistic arrangement. The individual strands in a β-sheet would be resistant to proteolytic digestion and the sheet might not unravel even if some of the loops connecting the strands were cleaved. Indeed, the connecting loops could be varied in size and sequence and still preserve the basic structure of the β-sheet (as is seen in the variable domains).

If one were to construct a binding site that could better withstand the action of digestive enzymes, one is well served to use the surface of a β-sheet. Moreover, the chances of preserving that site during evolution would be good, since such a site can better survive mutational events. In view of the stability of the sheet structure, about the only mutation that can significantly affect the binding site structure is one that alters a ligand-contacting residue; replacements of even the residues immediately adjacent to the ligand-contacting residues, unless drastic, may have little effect on the topography of the exposed surface.

On the other hand, if one were to construct a binding site that is deformable and that is easily varied (by mutation), one would choose to build it with loops. The structure of a loop is very sensitive to changes in length and sequence. Moreover, the residues in a loop are more likely to be exposed to solvent, and, thereby, are more available for ligand binding, than residues in a β-sheet. A survey of the exposures of the residues in the J539 and Kol V_L and V_H (computed in the context of isolated domains) reveals that the ratio of exposed (mE and Ex designations as defined in the footnote to Table 16) to buried (mB and Bu designations) residues is 0.70 when in the β-sheets (theoretically, this should be close to 1.0 since in an infinite sheet every other residue would be buried) and 1.72 when in the CDRs. Since some CDR residues are in fact in the sheets, the difference would have been even

Table 22. The framework residues in V_L which are in contact with CDRs or with V_H and those which are inward-pointing in murine Fabs of known three-dimensional structure

	10	20	35	40	49	60	70	80	88	98	107
	|			| | |||| |				| |	|||	
J539	EI.L.Q....T.A.....V.I.C		WYQQ....SP.PWIY		.V..RF.....T.Y.L.I..M...D.A.YYC		F...T.L.L.
McPC603	DIVMTQ....L.V.....V.M.C		WYQQ....PP.LLIY		.V.DRF....S.TDF.L.I..V...D.A.YYC		FGA.T.L.I.
HyHEL-10	DIVLTQ....L.V.....V.L.C	C	WYQQ....SP.LLIK	C	.I. RF....S.T.F.L.I..V...D..MYFC	C	F...T.L.I.
HyHEL-5	DIVL.Q....M.A.....V.M.C		WYQQ....SP.KWIY		.V..RF.....T.Y.L.I..M...D.A.YYC		FG..T.L...
R19.9	DIQMTQT...L.A.....V.I.C	D	WYQQ....TVKLLIY	D	.V..RF.....TDY.L.I..L...D.A.YFC	D	F.G.T.L...
4-4-20	DVVMTQ....L.V.....A.I.C		WYLQ....SPKVLIY		.V.DRF.....T.F.L.I..V...D...Y.C		F...T.L...
Yst9-1	DIQMTQ....L.A.....V.I.C	R	WYQQ...G.V.LLIY	R	.V..RF.....TDY.L.I..L...D.A.YIC	R	FG..T.L.I.
36-71	DIQM.Q....L.A.....V.I.C		WYQQ...G.I.LLIY		.V..RF.....TDY.L.I..L...D.A.YFC		F...T.L...
B13I2	DVLM.QT...L.V.....A.I.C		WYLQ....SP.LLIY		.V.DRFS.....TDF.L.I..V...D...YYC		F...T.L.I.
D1.3	DI.MTQ....L.A.....V.I.C	1	WYQQ....SP.LLVY	2	.V..RF....S.T.Y.L.I..L...DF..YYC	3	F.G.T.L...
BV04-01	.V.MTQ....L.V.....A.I.C		WYLQ...QSP.LLIY		.V..RF.....TDF.L.I..V...D...YFC		F.A.T.L...
AN02	QIVLTQ....M.A.....V.M.C		WYQQ..GSSP.LLIY		.V.VRF.....T.Y.L.I..M...D.A.YYC		F.V.T.L.L.
17/9	DIVMTQ....L.V.....V.M.C		WYQQ..G.PP.VLIY		.V.DRF....S.TDF.L.I..V...D.A.YYC		F...T.L...
8F5	DIVMTQ....L.V.....V.MTC		WYQQ....SP.LLIY		.V.DRFT...S.T.F.L.I..V...D.A.YYC		F...T.L.L.
MC41	DIVMTQ....M.T.....V.I.C		WYQQ....SP.LLIY		.V.DRF.....Y.L.I..V...D.A.YYC		F...T.L.I.
	^^^^^ ^ ^^		^^ ^^ ^^		^ ^ ^^ ^ ^^^ ^		^
			*				

Summary of the results presented in Tables 8, and 18–21.

|Residues which contact the opposite domain.

^Residues which contact CDRs.

*Residues which have been found to be involved in antigen binding.

Table 23. The framework residues in V_H which are in contact with CDRs or with V_L and those which are inward-pointing in murine Fabs of known three-dimensional structure

	10 20 30		40 49		70 82abc 90		102 110
J539	.V.L.E.....V.....L.L.C.A..FDFS		WVRQ...KGLEWI.		KF.I.R.N....L.L.M..V...D.A.YYCAR		WGQ.T.V.V..
McPC603	.V.L.E.....V.....L.L.C.T..FTFS		WVRQ....RLEWIA		RFIV.R.T....L.L.M..L...D.A.YYCAR		WG..T.V.V..
HyHEL-10	.V.L.E.....V.....L.L.C.V....IT	C	WIRK...N.LEYMG	C	RI.I.R.....Y.L.L..V...D.A.YYCAN	C	WG..T.V V..
HyHEL-5	...L.Q.....M.....V.I.C.A..YTFS		WVKQR...GLEWI.		KA.F.A.....A.M.LN.L...D...YYCLH		WG..T.L.V..
R19.9	.V.L.Q.....V.....V.M.C.A..YTFT	D	WVKQ....GLEWI.	D	KT.L.V.R....A.M.LR.L...D.A.YFCAR	D	WGQ.T.L.V..
4-4-20	...L.E.....V.....M.L.C.A..FTFS		WVRQS....LEWVA		RFTI.R.D..S.V.L.M..L...D...YYCT.		WGQ.T.V.V..
Ysr9-1	EV.L.E.....V.....L.L.C.T..FTFT	R	WVRQ....AL.WL.	R	RFTI.R.N....L.L.M..L...D.A.YYCTR	R	W.Q.T.V.V..
36-71	EV.L.Q.....V.....V.M.C.A..YTFT		WVKQ...Q.LEWI.		KT.L.V.K....A.M.L..L...D.A.YFCAR		W.Q.T.L.V..
B13I2	.V.L.E.....V.....L.L.C.A..FTFS		WVRQ...K.L.WVA		RF.I.R.N....L.L.M..L...D.A.YYCTR		WG..T.L.V..
D1.3	.V.L.E.....V.....L.I.C.V..F.LT	1	WVRQ....GLEWL.	2	RL.I.R.....V.L.M..L...D.A.YYCAR	3	WGQ.T.L.V..
BV04-01	E..P.E.....V.....L.L.C.A..FSFN		WVRQ...K.LEWVA		RF.I.R.D....L.L.M..L...D.A.YYCVR		WGQ.T.V.V..
AN02	.V.L.E.....V.....Q.L.C.V..YSIT		WIRQ...NKLEWM.		RISI.R.....F.L.LK.V...D.A.YFCAR		WGQ.T.V.V..
17/9	.V.L.E.....V.....L.L.C.A..FSFS		WVRQT..K.LEWVA		RFTI.R.N....L.L.M..L...D.A.YYCAR		WGQ.T.V.V..
8F5	.V.L.Q.....V.....V.L.C.T..F.IK		WVKQR...GLEWI.		KA.I.V.T....A.L.L..L...D.A.YYCD.		WG..T.V.V..
NC41	.I.L.Q.....K.....V.I.C.A..Y.FT		W.KQ....GLEWM.		RF.F.L.....A.L.I..L...D.A.FFCAR		WGQ.T.L.V..
	^^ ^ ^ ^^^^		^^^ ^ ^^^^		^^^^ ^ ^ ^ ^^ ^ ^^^		^
	*		*				

See footnote for Table 22.

Table 24. Solvent exposures of side chains of framework residues in Kol and J539 V_L

	Fractional accessibility					
	Kol			J539		
Position	Residue	Exposure		Residue	Exposure	
1	Q	1.00	Ex	E	0.99	Ex
2	S	1.00	Ex	I	0.16	Bu
3	V	0.77	mE	V	0.87	Ex
4	L	0.00	Bu	L	0.00	Bu
5	T	0.92	Ex	T	0.80	mE
6	Q	0.00	Bu	Q	0.00	Bu
7	P	0.62	mE	S	0.89	Ex
8	P	1.00	Ex	P	0.67	mE
9	—	—	—	A	1.00	Ex
10	S	1.00	Ex	I	0.94	Ex
11	A	0.34	mB	T	0.30	mB
12	S	0.71	mE	A	0.59	pB
13	G	1.00	Ex	A	0.00	Bu
14	T	0.73	mE	S	0.78	mE
15	P	0.75	mE	L	0.79	mE
16	G	1.00	Ex	G	1.00	Ex
17	Q	0.69	mE	Q	0.64	mE
18	R	0.79	mE	K	0.74	mE
19	V	0.21	mB	V	0.22	mB
20	T	0.62	mE	T	0.65	mE
21	I	0.00	Bu	I	0.00	Bu
22	S	0.92	Ex	T	0.69	mE
23	C	0.00	Bu	C	0.00	Bu
35	W	0.00	Bu	W	0.00	Bu
36	Y	0.00	Bu	Y	0.00	Bu
37	Q	0.46	pB	Q	0.14	Bu
38	Q	0.00	Bu	Q	0.24	mB
39	L	0.75	mE	K	0.69	mE
40	P	0.91	Ex	S	1.00	Ex
41	G	1.00	Ex	G	1.00	Ex
42	M	0.74	mE	T	0.90	Ex
43	A	0.62	mE	S	0.30	mB
44	P	0.00	Bu	P	0.00	Bu
45	K	0.95	Ex	K	0.90	Ex
46	L	0.23	mB	P	0.43	pB
47	L	0.15	Bu	W	0.16	Bu
48	I	0.00	Bu	I	0.00	Bu
49	Y	0.39	mB	Y	0.42	pB
57	G	1.00	Ex	G	1.00	Ex
58	V	0.14	Bu	V	0.13	Bu
59	P	0.70	mE	P	0.61	mE
60	D	0.95	Ex	A	1.00	Ex
61	R	0.31	mB	R	0.36	mB
62	F	0.12	Bu	F	0.00	Bu
63	S	0.85	Ex	S	0.94	Ex
64	G	0.00	Bu	G	0.00	Bu
65	S	1.00	Ex	S	1.00	Ex
66	K	0.41	pB	G	1.00	Ex
67	S	1.00	Ex	S	1.00	Ex
68	G	1.00	Ex	G	1.00	Ex
69	A	0.71	mE	T	0.75	mE
70	S	1.00	Ex	S	0.98	Ex
71	A	0.00	Bu	Y	0.09	Bu
72	S	1.00	Ex	S	0.70	mE
73	L	0.00	Bu	L	0.00	Bu
74	A	0.74	mE	T	0.43	pB
75	I	0.00	Bu	I	0.00	Bu
76	G	1.00	Ex	N	0.83	Ex
77	G	1.00	Ex	T	0.83	Ex
78	L	0.00	Bu	M	0.00	Bu
79	Q	0.76	mE	E	0.63	mE
80	S	1.00	Ex	A	0.96	Ex
81	E	0.78	mE	E	0.91	Ex
82	D	0.09	Bu	D	0.13	Bu
83	E	0.64	mE	A	0.55	pB
84	T	0.34	mB	A	0.00	Bu
85	D	0.30	mB	I	0.58	pB
86	Y	0.00	Bu	Y	0.00	Bu
87	Y	0.16	Bu	Y	0.11	Bu
88	C	0.00	Bu	C	0.00	Bu
98	F	0.04	Bu	F	0.00	Bu
99	G	0.00	Bu	G	1.00	Ex
100	T	0.59	pB	A	1.00	Ex
101	G	1.00	Ex	G	0.00	Bu
102	T	0.00	Bu	T	0.00	Bu
103	K	0.82	Ex	K	0.79	mE
104	V	0.00	Bu	L	0.00	Bu
105	T	0.86	Ex	E	0.89	Ex
106	V	0.19	Bu	L	0.44	pB
106a	L	0.70	mE	—	—	—
107	G	1.00	Ex	K	0.77	mE

See footnote to Table 16 for the definition of solvent accessibility.

more pronounced if only the CDR residues which are in loop regions were used in the comparison. Thus the mutation of a loop residue could have a profound effect on ligand binding, either directly, since by being more accessible the residue is more likely to be involved in the contact, or indirectly, since a change in its character could more easily affect the conformation of the local structure or that of the whole loop.

It comes as no surprise, therefore, that C1q binding, which is a desirable trait in all antibody types, (probably) involves residues in a sheet, while antigen binding involves loops.

One wonders whether deformability is built into (some) CDR structures. Deformability of the combining site structure may permit "induced fit", which could result in better binding to ligand. Deformability may also result in the capacity to bind to many different ligands (polyreactivity). The ability to react to a greater variety of antigens using fewer antibodies, albeit with low affinity, affords an organism a distinct survival advantage. The observation that the occurrence of glycines in CDR3-H is a natural consequence of the preferred mode of transcription of D-gene segments (Abergel and Claverie, 1991) is of relevance. In view of the fact that glycines can confer flexibility to a polypeptide segment and that CDR3-H is often observed to play a central role in ligand binding, a deformable combining site may be the norm. It would be interesting to compare pre-immune and mature antibodies in this respect. Are pre-immune antibodies more deformable than mature ones? Is the rigidification of the combining site structure a factor in affinity maturation?

An increased rigidity of the combining site can be achieved by reducing the inherent flexibility of the individual CDRs (by reducing the number of glycines,

Table 25. Solvent exposures of side chains of framework residues in Kol and J539 V_H

Position	Kol Residue	Fractional accessibility Kol Exposure		J539 Residue	Fractional accessibility J539 Exposure	
1	E	1.00	Ex	E	1.00	Ex
2	V	0.23	mB	V	0.37	mB
3	Q	0.82	Ex	K	0.82	Ex
4	L	0.00	Bu	L	0.10	Bu
5	V	0.87	Ex	L	1.00	Ex
6	Q	0.00	Bu	E	0.09	Bu
7	S	0.94	Ex	S	0.94	Ex
8	G	1.00	Ex	G	1.00	Ex
9	G	0.00	Bu	G	0.00	Bu
10	G	1.00	Ex	G	1.00	Ex
11	V	0.90	Ex	L	0.81	Ex
12	V	0.25	mB	V	0.25	mB
13	Q	0.71	mE	Q	0.87	Ex
14	P	0.59	pB	P	0.64	mE
15	G	1.00	Ex	G	1.00	Ex
16	R	0.73	mE	G	1.00	Ex
17	S	0.66	mE	S	0.75	mE
18	L	0.28	mB	L	0.26	mB
19	R	0.66	mE	K	0.75	mE
20	L	0.00	Bu	L	0.00	Bu
21	S	0.71	mE	S	0.82	Ex
22	C	0.00	Bu	C	0.00	Bu
23	S	1.00	Ex	A	1.00	Ex
24	S	0.00	Bu	A	0.00	Bu
25	S	0.87	Ex	S	1.00	Ex
26	G	1.00	Ex	G	1.00	Ex
27	F	0.10	Bu	F	0.10	Bu
28	I	0.85	Ex	D	0.72	mE
29	F	0.00	Bu	F	0.00	Bu
30	S	0.74	mE	S	0.83	Ex
36	W	0.00	Bu	W	0.00	Bu
37	V	0.00	Bu	V	0.00	Bu
38	R	0.10	Bu	R	0.31	mB
39	Q	0.15	Bu	Q	0.28	mB
40	A	0.95	Ex	A	0.75	mE
41	P	0.90	Ex	P	0.73	mE
42	G	1.00	Ex	G	1.00	Ex
43	K	0.86	Ex	K	0.86	Ex
44	G	1.00	Ex	G	1.00	Ex
45	L	0.00	Bu	L	0.00	Bu
46	E	0.75	mE	E	0.73	mE
47	W	0.10	Bu	W	0.04	Bu
48	V	0.00	Bu	I	0.00	Bu
49	A	0.00	Bu	G	0.00	Bu
66	R	0.36	mB	K	0.51	pB
67	F	0.00	Bu	F	0.00	Bu
68	T	0.87	Ex	I	0.88	Ex
69	I	0.00	Bu	I	0.00	Bu
70	S	0.78	mE	S	0.79	mE
71	R	0.11	Bu	R	0.00	Bu
72	N	0.61	mE	D	0.55	pB
73	D	0.44	pB	N	0.43	pB
74	S	0.85	Ex	A	0.97	Ex
75	K	0.88	Ex	K	0.77	mE
76	N	0.69	mE	N	0.68	mE
77	T	0.41	pB	S	0.33	mB
78	L	0.00	Bu	L	0.00	Bu
79	F	0.45	pB	Y	0.35	mB
80	L	0.00	Bu	L	0.00	Bu
81	Q	0.53	pB	Q	0.69	mE
82	M	0.00	Bu	M	0.00	Bu
82a	D	0.73	mE	S	0.58	pB
82b	S	0.98	Ex	K	0.96	Ex
82c	L	0.00	Bu	V	0.00	Bu
83	R	0.73	mE	R	0.83	Ex
84	P	0.75	mE	S	0.90	Ex
85	E	0.82	Ex	E	0.90	Ex
86	D	0.00	Bu	D	0.11	Bu
87	T	0.54	pB	T	0.47	pB
88	G	1.00	Ex	A	0.00	Bu
89	V	0.58	pB	L	0.63	mE
90	Y	0.00	Bu	Y	0.00	Bu
91	F	0.00	Bu	Y	0.08	Bu
92	C	0.00	Bu	C	0.00	Bu
93	A	0.00	Bu	A	0.00	Bu
94	R	0.17	Bu	R	0.15	Bu
103	W	0.09	Bu	W	0.07	Bu
104	G	0.00	Bu	G	1.00	Ex
105	Q	0.93	Ex	Q	0.99	Ex
106	G	0.00	Bu	G	0.00	Bu
107	T	0.22	mB	T	0.26	mB
108	P	0.99	Ex	L	0.67	mE
109	V	0.00	Bu	V	0.00	Bu
110	T	0.76	mE	T	0.69	mE
111	V	0.00	Bu	V	0.00	Bu
112	S	0.98	Ex	S	0.74	mE
113	S	0.94	Ex	A	0.84	Ex

See footnote to Table 16 for the definition of solvent accessibility.

increasing the number of prolines or asparagines, by shortening the CDR, etc), by increasing the interaction of the CDRs or of the individual CDR residues with surrounding structures (framework and other CDRs), by replacing critical CDR residues with amino acids having fewer degrees of freedom, and/or by strengthening the V_L–V_H interaction. In this regard, it is interesting to note that the residues which have been replaced during the maturation of the anti-2-phenyloxazolone response, the ligand-contacting residues at positions 34 and 36 of the light chain (Alzari *et al.*, 1990), are also frequently found to be involved in the V_L–V_H contact (Table 8a).

Another contact that deserves mention is that between framework and CDRs within each domain. The observation that a framework residue can influence the conformation of a CDR implies that differences in framework structure, albeit small, could have a significant effect on the specificity of the combining site. This could explain in part why particular variable region families or subgroups are found to be associated with certain antigen-binding specificities. Conversely, particular CDR–framework combinations may not be favorable and may not be formed (see also Kirkham *et al.*, 1992).

Acknowledgements—I thank my colleagues, Drs Chantal Abergel and Jennifer Tipper, for their critical reading of the manuscript and for comments and discussion, and Dr Allen B. Edmundson and coworkers for kindly providing atomic coordinates for BV04-01.

Table 26. Various enzyme–polypeptide inhibitor interactions

PDB Code	Enzyme	Surf. buried	Ligand	Surf. buried	vdW	m.c.vdW	H.b.	m.c. H.b.	I.p.
1CSE	Subtilisin Carlsberg	667	Eglin C	667	162	75	16	7	0
3TEC	Thermitase	674	Eglin C	670	158	64	16	7	0
2PTC	β-trypsin	714	BPTI	588	170	78	16	5	0
2SNI	Subtilisin novo	776	Chyt. inhib. (barley)	742	180	70	13	9	0
1CHO	α-Chyt.	701	Ovomucoid (turkey)	628	138	60	12	8	0
2KAI	Kallikrein A	694	BPTI	606	179	77	14	6	0
3SGB	Proteinase B (S. Griseus)	571	Ovomucoid (turkey)	545	144	67	11	7	0
4SGB	Proteinase B (S. Griseus)	578	Chyt. inhib. (potato)	517	126	62	9	5	0
1TAB	Trypsin	662	Prot. inhib. (Bowman-Birk)	584	134	69	13	5	1
4CPA	Carboxypep. A	598	Carb. inhib. (potato)	483	96	21	9	1	1
3APR	Rhizopuspep.	709	Peptide inhibitor	551	145	36	9	5	0
4ER4	Endothiapep.	814	Peptide inhibitor	696	168	54	10	5	1

See footnote to Table 4 for the definition of contacts.

REFERENCES

Abola E. E., Bernstein F. C., Bryant S. H., Koetzle T. F. and Weng J. (1987) Protein Data Bank. In *Crystallographic Databases—Information Content, Software Systems, Scientific Application* (Edited by Allen F. C., Bergerhoff G. and Sievers R.), pp. 107–132. Data Commission of the International Union of Crystallography, Bonn/Cambridge/Chester.

Abergel C. and Claverie J.-M. (1991) A strong propensity toward loop formation characterizes the expressed reading frames of the D segments at the Ig H and T cell receptor loci. *Eur. J. Immunol.* **21**, 3021–3025.

Aitschuh D., Vix O., Rees B. and Thierry J.-C. (1992) A conformation of cyclosporin A in aqueous environment revealed by the X-ray structure of a cyclosporin-Fab complex. *Science* **256**, 92–94.

Alzari P. M., Lascombe M.-B. and Poljak R. J. (1988) Three-dimensional structure of antibodies. *A. Rev. Immunol.* **6**, 555–580.

Alzari P. M., Spinelli S., Mariuzza R. A., Boulot G., Poljak R. J., Jarvis J. M. and Milstein C. (1990) Three-dimensional structure determination of an anti-2-phenyloxazolone antibody: the role of somatic mutation and heavy/light chain pairing in the maturation of an immune system. *Eur. molec. Biol. Org. J.* **9**, 3807–3814.

Amit A. G., Mariuzza R. A., Phillips S. E. V. and Poljak R. J. (1986) Three-dimensional structure of an antigen-antibody complex at 2.8 Å resolution. *Science* **233**, 747–753.

Amzel L. M. and Poljak R. J. (1979) Three-dimensional structure of immunoglobulins. *A. Rev. Biochem.* **48**, 961–997.

Amzel L. M., Poljak R. J., Saul F., Varga J. M. and Richards F. F. (1974) The three dimensional structure of a combining region-ligand complex of immunoglobulin NEW at 3.5-A resolution. *Proc. natn. Acad. Sci. U.S.A.* **71**, 1427–1430.

Anglister J. (1990) Use of deuterium labelling in NMR studies of antibody: combining site structure. *Q. Rev. Biophys.* **23**, 173–203.

Arnold E., Jacobo-Molina A., Nanni R. G., Williams R. L., Lu X., Ding J., Clark A. D., Jr, Zhang A., Ferris A. L., Clark P., Hizi A. and Hughes S. H. (1992) Structure of HIV-1 reverse transcriptase–DNA complex at 7 Å resolution showing active site locations. *Nature* **357**, 85–89.

Baccala R., Quang T. V., Gilbert M., Ternynck T. and Avrameas S. (1989) Two murine natural polyreactive autoantibodies are encoded by nonmutated germ-line genes. *Proc. natn. Acad. Sci. U.S.A.* **86**, 4624–4628.

Beale D. and Feinstein A. (1976) Structure and function of the constant regions of immunoglobulins. *Q. Rev. Biophys.* **9**, 135–180.

Benjamin D. C., Berzofsky J. A., East I. J., Gurd F. R. N., Hannum C., Leach S. J., Margoliash E., Michael J. G., Miller A., Prager E. M., Reichlin M., Sercarz E. E., Smith-Gill S. J., Todd P. E. and Wilson A. C. (1984) The antigenic structure of proteins: a reappraisal. *A. Rev. Immunol.* **2**, 67–101.

Benkovic S. J. (1992) Catalytic antibodies. *A. Rev. Biochem.* **61**, 29–54.

Bentley G. A., Boulot G., Riottot M. M. and Poljak R. J. (1990) Three-dimensional structure of an idiotope–anti-idiotope complex. *Nature* **348**, 254–257.

Bernstein F. C., Koetzle T. F., Williams G. J. B., Meyer E. F., Jr, Brice M. D., Rodgers J. R., Kennard O., Shimanouchi T. and Tasumi M. (1977) The Protein Data Bank. A computer-based archival file for macromolecular structures. *. molec. Biol.* **112**, 535–542.

Bhat T. N., Bentley G. A., Fischmann T. O., Boulot G. and Poljak R. J. (1990) Small rearrangements in structures of Fv and Fab fragments of antibody D1.3 on antigen binding. *Nature* **347**, 483–485.

Bird R. E., Hardman K. D., Jacobson J. W., Johnson S., Kaufman B. M., Lee S.-M., Lee T., Pope S. H., Riordan G. S. and Whitlow M. (1988) Single-chain antigen-binding proteins. *Science* **242**, 423–426.

Bizebard T., Mauguen Y., Skehel J. J. and Knossow M. (1991) Use of molecular replacement in the solution of an immunoglobulin Fab fragment structure. *Acta Cryst.* **B47**, 549–555.

Brady R. L., Edwards D. J., Hubbard R. E., Jiang J.-S., Lange G., Roberts S. M., Todd R. J., Adair J. R., Emtage J. S., King D. J. and Low D. C. (1992) Crystal structure of a chimeric Fab' fragment of an antibody binding tumour cells. *J. molec. Biol.* **227**, 253–264.

Bruenger A. T. (1991) Solution of a Fab (26-10)/digoxin complex by generalized molecular replacement. *Acta Cryst.* **A47**, 195–204.

Bruenger A. T., Leahy D. J., Hynes T. R. and Fox R. O. (1991) 2.9 Å resolution structure of an anti-dinitrophenyl-spin-label monoclonal antibody Fab fragment with bound hapten. *J. molec. Biol.* **221**, 239–256.

Bryant S. H., Amzel L. M., Phizackerley R. P. and Poljak R. J. (1985) Molecular-replacement structure of guinea pig IgG1 pFc′ refined at 3.1 Å resolution. *Acta Cryst.* **B41**, 362–368.

Burastero S. E., Casali P., Wilder R. L. and Notkins A. B. (1988) Monoreactive high affinity and polyreative low affinity rheumatoid factors are produced by CD5+ B cells from patients with rheumatoid arthritis. *J. exp. Med.* **168**, 1979–1992.

Burton D. R. (1985) Immunoglobulin G: functional sites. *Molec. Immun.* **22**, 161–206.

Burton D. R. (1990*a*) The conformation of antibodies. In *Fc Receptors And The Action of Antibodies* (Edited by Metzger H.), pp. 31–54. American Society for Microbiology, Washington, D.C.

Burton D. R. (1990*b*) Antibody: the flexible adaptor molecule. *Trends biochem. Sci.* **15**, 64–69.

Carter P., Presta L., Gorman C. M., Ridgway J. B. B., Henner D., Wong W. L. T., Rowland A. M., Kotts C., Carver M. E. and Shepard H. M. (1992) Humanization of an anti-p185^{HER2} antibody for human cancer therapy. *Proc. natn. Acad. Sci. U.S.A.* **89**, 4285–4289.

Chang C.-H., Short M. T., Westholm F. A., Stevens F. J., Wang B.-C., Furey W., Jr, Solomon A. and Schiffer M. (1985) Novel arrangement of immunoglobulin variable domains: X-ray crystallographic analysis of the λ-chain dimer Bence–Jones protein Loc. *Biochemistry* **24**, 4890–4897.

Chaudary V. K., Queen C., Junghans R. P., Waldmann T. A., Fitzgerald D. J. and Pastan I. (1989) A recombinant immunotoxin consisting of two antibody variable domains fused to *Pseudomonas* exotoxin. *Nature* **339**, 394–397.

Chothia C. and Lesk A. M. (1987) Canonical structures for the hypervariable regions of immunoglobulins. *J. molec. Biol.* **196**, 901–917.

Chothia C., Lesk A. M., Gherardi E., Tomlinson I. M., Walter G., Marks J. D., Llewyn M. B. and Winter G. (1992) Structural repertoire of the human V_H segments. *J. molec. Biol.* **227**, 799–817.

Chothia C., Lesk A. M., Tramontano A., Levitt M., Smith-Gill S. J., Air G., Sheriff S., Padlan E. A., Davies D., Tulip W. R., Colman P. M., Spinelli S., Alzari P. M. and Poljak R. J. (1989) Conformation of immunoglobulin hypervariable regions. *Nature* **342**, 877–883.

Chothia C., Novotny J., Bruccoleri R. and Karplus M. (1986) Domain association in immunoglobulin molecules. The packing of variable domains. *J. molec. Biol.* **186**, 651–663.

Co M. S. and Queen C. (1991) Humanized antibodies for therapy. *Nature* **351**, 501–502.

Colcher D., Minelli M. F., Roselli M., Muraro R., Simpson-Milenic D. and Schlom J. (1988) Radioimmunolocalization of human carcinoma xenografts with B72.3 second generation (CC) monoclonal antibodies. *Cancer Res.* **48**, 4597–4603.

Colman P. M. (1988) Structure of antibody-antigen complexes: implications for immune recognition. *Adv. Immunol.* **43**, 99–132.

Colman P. M., Laver W. G., Varghese J. N., Baker A. T., Tulloch P. A., Air G. M. and Webster R. G. (1987) Three-dimensional structure of a complex of antibody with influenza virus neuraminidase. *Nature* **326**, 358–363.

Colman P. M., Schramm H. J. and Guss J. M. (1977) Crystal and molecular structure of the dimer of variable domains of the Bence–Jones protein ROY. *J. molec Biol.* **116**, 73–79.

Colman P. M., Tulip W. R., Varghese J. N., Tulloch P. A., Baker A. T., Laver W. G., Air G. M. and Webster R. G. (1989) Three-dimensional structures of influenza virus neuraminidase–antibody complexes. *Phil. Trans. R. Soc. Lond. B* **323**, 511–518.

Connolly M. L. (1983) Analytical molecular surface calculation. *J. appl. Crystallogr.* **16**, 548–558.

Cygler M., Boodhoo A., Lee J. S. and Anderson W. F. (1987) Crystallization and structure determination of an autoimmune anti-poly(dT) immunoglobulin Fab fragment at 3.0 A resolution. *J. biol. Chem.* **262**, 643–648.

Cygler M., Rose D. R. and Bundle D. R. (1991) Recognition of a cell-surface oligosaccharide of pathogenic *Salmonella* by an antibody Fab fragment. *Science* **253**, 442–445.

Davies D. R. and Chacko S. (1993) Antibody structure. *Acc. Chem. Res.* **26**, 421–427.

Davies D. R. and Metzger H. (1983) Structural basis of antibody function. *A. Rev. Immunol.* **1**, 87–117.

Davies D. R. and Padlan E. A. (1992) Twisting into shape. *Curr. Biol.* **2**, 254–256.

Davies D. R., Padlan E. A. and Segal D. M. (1975*a*) Three-dimensional structure of immunoglobulins. *A. Rev. Biochem.* **44**, 639–667.

Davies D. R., Padlan E. A. and Segal D. M. (1975*b*) Immunoglobulin structures at high resolution. In *Contemporary Topics In Molecular Immunology*, Vol. 4 (Edited by Inman F. P. and Mandy W. J.), pp. 127–155. Plenum Press, New York.

Davies D. R., Padlan E. A. and Sheriff S. (1990) Antibody-antigen complexes. *A. Rev. Biochem.* **59**, 439–473.

Davies D. R., Sheriff S. and Padlan E. A. (1988) Antibody/antigen complexes. *J. biol. Chem.* **263**, 10541–10544.

Deisenhofer J. (1981) Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of Protein A from *Staphylococcus aureus* at 2.9- and 2.8-Å resolution. *Biochemistry* **20**, 2361–2370.

De la Paz P., Sutton B. J., Darsley M. J. and Rees A. R. (1986) Modeling of the combining sites of three anti-lysozyme monoclonal antibodies and of the complex between one of the antibodies and its epitope. *Eur. molec. Biol. Org. J.* **5**, 415–425.

Derrick J. P. and Wigley D. B. (1992) Crystal structure of a streptococcal protein G domain bound to an Fab fragment. *Nature* **359**, 752–754.

Duncan A. R. and Winter G. (1988) The binding site for C1q on IgG. *Nature* **332**, 738–740.

Duncan A. R., Woof J. M., Partridge L. J., Burton D. R. and Winter G. (1988) Localization of the binding site for the human high-affinity Fc receptor for IgG. *Nature* **332**, 563–564.

Edelman G. M., Cunningham B. A., Gall W. E., Gottlieb P. D., Rutishauser U. and Waxdal M. J. (1969) The covalent structure of an entire γG immunoglobulin molecule. *Proc. natn. Acad. Sci. U.S.A.* **63**, 78–85.

Edmundson A. B., Ely K. R. and Abola E. E. (1978) Conformational flexibility in immunoglobulins. In *Contemporary Topics In Molecular Immunology*, Vol. 7 (Edited by Reisfeld R. A. and Inman F. P.), pp. 95–118. Plenum Press, New York.

Edmundson A. B., Ely K. R., Girling R. L., Abola E. E., Schiffer M., Westholm F. A., Fausch M. D. and Deutsch H. F. (1974) Binding of 2,4-dinitrophenyl compounds and other small molecules to a crystalline λ-type Bence–Jones dimer. *Biochemistry* **13**, 3816–3826.

Edmundson A. B., Ely K. R., Herron J. N. and Cheson B. D. (1987) The binding of opioid peptides to the Mcg light chain dimer: flexible keys and adjustable locks. *Molec. Immun.* **24**, 915–935.

Eigenbrot C., Randal M., Presta L., Carter P. and Kossiakoff A. A. (1993) X-ray structures of the antigen-binding domains from three variants of humanized anti-p185^{HER2} antibody 4D5 and comparison with molecular modeling. *J. molec. Biol.* **229**, 969–995.

Ely K. R., Herron J. N., Harker M. and Edmundson A. B. (1989) Three-dimensional structure of a light chain dimer crystallized in water. Conformational flexibility of a molecule in two crystal forms. *J. molec. Biol.* **210**, 601–615.

Ely K. R., Wood M. K., Rajan S. S., Hodsdon J. M., Abola E. E., Deutsch H. F. and Edmundson A. B. (1985) Unexpected similarities in the crystal structures of the Mcg light chain dimer and its hybrid with the Weir protein. *Molec. Immun.* **22**, 93–100.

Epp O., Colman P., Fehlhammer H., Bode W., Schiffer M., Huber R. and Palm W. (1974) Crystal and molecular structure of a dimer composed of the variable portions of the Bence-Jones protein REI. *Eur. J. Biochem.* **45**, 513–524.

Epp O., Lattman E. E., Schiffer M., Huber R. and Palm W. (1975) The molecular structure of a dimer composed of the variable portions of the Bence–Jones protein REI refined at 2.0 Å resolution. *Biochemistry* **14**, 4943–4952.

Fan Z.-C., Shan L., Guddat L. W., He X.-M., Gray W. R., Raison R. L. and Edmundson A. B. (1992) Three-dimensional structure of an Fv from a human IgM immunoglobulin. *J. molec. Biol.* **228**, 188–207.

Fehlhammer H., Schiffer M., Epp O., Colman P. M., Lattman E. E., Schwager P., Steigemann W. and Schramm H. J. (1975) The structure determination of the variable portion of the Bence–Jones protein Au. *Biophys. Struct. Mechanism* **1**, 139–146.

Fersht A. R., Shi J.-P., Knill-Jones J., Lowe D. M., Wilkinson A. J., Blow D. M., Brick P., Carter P., Wayne M. M. and Winter G. (1985) Hydrogen bonding and biological specificity analysed by protein engineering. *Nature* **314**, 235–238.

Fischmann T. O., Bentley G. A., Bhat T. N., Boulot G., Mariuzza R. A., Phillips S. E. V., Tello D. and Poljak R. J. (1991) Crystallographic refinement of the three-dimensional structure of the FabD1.3-lysozyme complex at 2.5-Å resolution. *J. biol. Chem.* **266**, 12915–12920.

Foote J. and Winter G. (1992) Antibody framework residues affecting the conformation of the hypervariable loops. *J. molec. Biol.* **224**, 487–499.

Furey W., Jr, Wang B. C., Yoo C. S. and Sax M. (1983) Structure of a novel Bence–Jones protein (Rhe) fragment at 1.6 Å resolution. *J. molec. Biol.* **167**, 661–692.

Garcia K. C., Ronco P. M., Verroust P. J., Bruenger A. T. and Amzel L. M. (1992) Three-dimensional structure of an angiotensin II-Fab complex at 3 Å: hormone recognition by an anti-idiotypic antibody. *Science* **257**, 502–507.

Glockshuber R., Schmidt T. and Plueckthun A. (1992) The disulfide bonds in antibody variable domains: effects on stability, folding *in vitro* and function expression in *Escherichia coli*. *Biochemistry* **31**, 1270–1279.

Gonzalez-Quintial R., Baccala R., Alzari P. M., Nahmias C., Mazza G., Fougereau M. and Avrameas S. (1990) Poly(Glu^{60}Ala(30Tyr^{10}) (GAT)-induced IgG monoclonal antibodies cross-react with various self and non-self antigens through the complementary determining regions. Comparison with IgM monoclonal polyreactive natural antibodies. *Eur. J. Immunol.* **20**, 2383–2387.

Harris L. J., Larson S. B., Hasel K. W., Day J., Greenwood A. and McPherson A. (1992) The three-dimensional structure of an intact monoclonal antibody for canine lymphoma. *Nature* **360**, 369–372.

He X. M., Rueker F., Casale E. and Carter D. C. (1992) Structure of a human monoclonal antibody Fab fragment against gp41 of human immunodeficiency virus type 1. *Proc. natn. Acad. Sci. U.S.A.* **89**, 7154–7158.

Helm B. A. (1989) The interaction of human IgE with class-specific Fc-receptors. In *Advances in the Biosciences*, Vol. 74 (Edited by Merret T. and El Shami A. L.), pp. 83–91. Pergamon Press, London.

Helm B. A., Ling. Y., Teale C., Padlan E. A. and Brueggemann M. (1991) The nature and biological importance of the inter-ε chain disulfide bonds in human IgE. *Eur. J. Immunol.* **21**, 1543–1548.

Herron J. N., He X. M., Ballard D. W., Blier P. R., Pace P. E., Bothwell A. L. M., Voss E. W., Jr and Edmundson A. B. (1991) An autoantibody to single-stranded DNA: comparison of the three-dimensional structures of the unliganded Fab and a deoxynucleotide-Fab complex. *PROTEINS: Struc. Funct. Genet.* **11**, 159–175.

Herron J. N., He X.-M., Mason M. L., Voss E. W., Jr and Edmundson A. B. (1989) Three-dimensional structure of a fluorescein-Fab complex crystallized in 2-methyl-2,4-pentanediol. *PROTEINS: Struc. Funct. Genet.* **5**, 271–280.

Huber R. (1986) Structural basis for antigen–antibody recognition. *Science* **233**, 702–703.

Huber R. and Bennett W. S. (1987) Antibody–antigen flexibility. *Nature* **326**, 334–335.

Huston J. S., Levinson D., Mudgett-Hunter M., Tai M.-S., Novotny J., Margolies M. N., Ridge R. J., Bruccoleri R. E., Haber E., Crea R. and Oppermann H. (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc. natn. Acad. Sci. U.S.A.* **85**, 5879–5883.

Janin J. and Chothia C. (1990) The structure of protein–protein recognition sites. *J. biol. Chem.* **265**, 16,027–16,030.

Jeffrey G. A. and Saenger W. (1991) *Hydrogen Bonding in Biological Structures*. Springer, Berlin.

Jencks W. P. (1969) *Catalysis in Chemistry And Enzymology*. McGraw-Hill, New York.

Jin L., Fendly B. M. and Wells J. A. (1992) High resolution functional analysis of antibody–antigen interactions. *J. molec. Biol.* **226**, 851–865.

Jones P. T., Dear P. H., Foote J., Neuberger M. S. and Winter G. (1986) Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature* **321**, 522–525.

Kabat E. A., Wu T. T. and Bilofsky H. (1977) Unusual distribution of amino acids in complementarity-determining (hypervariable) segments of heavy and light chains of immunoglobulins and their possible roles in specificity of antibody combining sites. *J. biol. Chem.* **252**, 6609–6616.

Kabat E. A., Wu T. T. and Bilofsky H. (1978) Variable region genes for the immunoglobulin framework are assembled from small segments of DNA—a hypothesis. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2429–2433.

Kabat E. A., Wu T. T., Perry H. M., Gottesman K. S. and Foeller C. (1991) *Sequences of Proteins of Immunological Interest*., 5th Edition, US Department of Health and Human Services, Public Health Service, National Institutes of Health (NIH Publication No 91-3242).

Kabsch W. and Sanders C. (1983) Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers* **22**, 2577–2637.

Kirkham P. M., Mortari F., Newton J. A. and Schroeder H. W., Jr (1992) Immunoglobulin V_H clan and family identity predicts variable domain structure and may influence antigen binding. *Eur. molec. Biol. Org. J.* **11**, 603–609.

Koehler G. and Milstein C. (1975) Continuous cultures of fused cells secreting antibodies of predefined specificity. *Nature* **256**, 495–497.

Lascombe M.-B., Alzari P. M., Poljak R. J. and Nisonoff A. (1992) Three-dimensional structure of two crystal forms of FabR19.9 from a monoclonal anti-arsonate antibody. *Proc. natn. Acad. Sci. U.S.A.* **89**, 9429–9433.

Lerner R. A. and Tramontano A. (1988) Catalytic antibodies. *Sci. Amer.* **258**(3), 58–70.

Lescar J., Riottot M.-M., Souchon H., Chitarra V., Bentley G. A., Navaza J., Alzari P. M. and Poljak R. J. (1993) Crystallization, preliminary X-ray diffration study, and crystal packing of a complex between anti-hen lysozyme antibody F9.14.7 and guinea-fowl lysozyme. *PROTEINS: Struct. Funct. Genet.* **15**, 209–212.

Lesk A. M. and Chothia C. (1988) Elbow motion in the immunoglobulins involves a molecular ball-and-socket joint. *Nature* **335**, 188–190.

Levitt M. and Perutz M. F. (1988) Aromatic rings act as hydrogen bond acceptors. *J. molec. Biol.* **201**, 751–754.

Levy R., Assulin O., Scherf T., Levitt M. and Anglister J. (1989) Probing antibody diversity by 2D NMR: comparison of amino acid sequences, predicted structures, and observed antibody–antigen interactions in complexes of two antipeptide antibodies. *Biochemistry* **28**, 7168–7175.

Logtenberg T. (1990) Properties of polyreactive natural antibodies to self and foreign antigens. *J. clin. Immunol.* **10**, 137–140.

Luzzati V. (1953) Resolution d'une structure cristalline lorsque les positions d'une partie des atomes sont connues: traitment statistique. *Acta Cryst.* **6**, 142–152.

Mariuzza R. A., Phillips S. E. V. and Poljak R. J. (1987) The structural basis of antigen–antibody recognition. *A. Rev. Biophys. Chem.* **16**, 139–159.

Marquart M., Deisenhofer J., Huber R. and Palm W. (1980) Crystallographic refinement and atomic models of the intact immunoglobulin molecule Kol and its antigen-binding fragment at 3.0 A and 1.9 A resolution. *J. molec. Biol.* **141**, 369–391.

Mian I. S., Bradwell A. R. and Olson A. J. (1991) Structure, function and properties of antibody binding sites. *J. molec. Biol.* **217**, 133–151.

Miller S. (1990) Protein–protein recognition and the association of immunoglobulin constant domains. *J. molec. Biol.* **216**, 965–973.

Morrison S. L., Johnson M. J., Herzenberg L. A. and Oi V. T. (1984) Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. *Proc. natn. Acad. Sci. U.S.A.* **81**, 6851–6855.

Morrison S. L. and Oi V. T. (1988) Genetically-engineered antibody molecules. *Adv. Immunol.* **44**, 65–92.

Novotny J., Bruccoleri R., Newell J., Murphy D., Haber E. and Karplus M. (1983) Molecular anatomy of the antibody binding site. *J. biol. Chem.* **258**, 14,433–14,437.

Novotny J., Bruccoleri R. E. and Saul F. A. (1989) On the attribution of binding energy in antigen–antibody complexes McPC603, D1.3 and HyHEL-5. *Biochemistry* **28**, 4735–4749.

Novotny J. and Haber E. (1985) Structural invariants of antigen binding: comparison of immunoglobulin V_L–V_H and V_L–V_L domain dimers. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4592–4596.

Novotny J. and Sharp K. (1992) Electrostatic fields in antibodies and antibody/antigen complexes. *Prog. Biophys. molec. Biol.* **58**, 203–224.

Padlan E. A. (1977*a*) Structural basis for the specificity of antibody–antigen reactions and structural mechanisms for the diversification of antigen-binding specificities. *Q. Rev. Biophys.* **10**, 35–65.

Padlan E. A. (1977*b*) Structural implications of sequence variability in immunoglobulins. *Proc. natn. Acad. Sci. U.S.A.* **74**, 2551–2555.

Padlan E. A. (1990*a*) On the nature of antibody combining sites: unusual features that may confer on these sites an enhanced capacity for binding ligands. *PROTEINS: Struct. Funct. Genet.* **7**, 112–124.

Padlan E. A. (1990*b*) X-ray diffraction studies of antibody constant regions. In *Fc Receptors And The Action Of Antibodies* (Edited by Metzger H.), pp. 12–30. American Society for Microbiology, Washington, D.C.

Padlan E. A. (1991) A possible procedure for reducing the immunogenicity of antibody variable domains while preserving their ligand-binding properties. *Molec. Immun.* **28**, 489–498.

Padlan E. A. (1992) Structure of protein epitopes deduced from X-ray crystallography. In *Structure of Antigens*, Vol. 1 (Edited by Van Regenmortel M. H. V.), pp. 29–42. CRC Press, Boca Raton.

Padlan E. A., Cohen G. H. and Davies D. R. (1985) On the specificity of antibody/antigen interactions: phosphocholine binding to McPC603 and the correlation of three-dimensional structure and sequence data. *A. Inst. Pasteur/Immunol.* **136C**, 271–276.

Padlan E. A., Cohen G. H. and Davies D. R. (1986) Antibody Fab assembly: the interface residues between CH1 and CL. *Molec. Immun.* **23**, 951–960.

Padlan E. A. and Davies D. R. (1975) Variability of three-dimensional structure in immunoglobulins. *Proc. natn. Acad. Sci. U.S.A.* **72**, 819–823.

Padlan E. A. and Davies D. R. (1986) A model of the Fc of immunoglobulin E. *Molec. Immun.* **23**, 1063–1075.

Padlan E. A., Davies D. R., Rudikoff S. and Potter. M. (1976) Structural basis for the specificity of phosphorylcholine-binding immunoglobulins. *Immunochemistry* **13**, 945–949.

Padlan E. A., Segal D. M., Spande T. F., Davies D. R., Rudikoff R. and Potter M. (1973) Structure at 4.5 Å resolution of a phosphorylcholine-binding Fab. *Nature New Biol.* **245**, 165–167.

Padlan E. A., Silverton E. W., Sheriff S., Cohen G. H., Smith-Gill S. J. and Davies D. R. (1989) Structure of an antibody–antigen complex: crystal structure of the HyHEL-10 Fab-lysozyme complex. *Proc. natn. Acad. Sci. U.S.A.* **86**, 5938–5942.

Perkins S. J., Nealis A. S., Sutton B. J. and Feinstein A. (1991) Solution structure of human and mouse immunoglobulin M by synchrotron X-ray scattering and molecular graphics

modeling. A possible mechanism for complement activation. *J. molec. Biol.* **221**, 1345–1366.

Poljak R. J., Amzel L. M., Chen B. L., Phizackerley R. P. and Saul F. (1975*a*) Structural basis for the association of heavy and light chains and the relation of subgroups to the conformation of the active site of immunoglobulins. *Immunogenetics* **2**, 393–394.

Poljak R. J., Amzel L. M., Chen B. L., Phizackerley R. P. and Saul F. (1975*b*) Structure and specificity of antibody molecules. *Phil. Trans. R. Soc. Lond. B.* **272**, 43–51.

Poljak R. J., Amzel L. M. and Phizackerley R. P. (1976) Studies on the three-dimensional structure of immunoglobulins. *Prog. Biophys. molec. Biol.* **31**, 67–93.

Poljak R. J., Amzel L. M., Avey H. P., Chen B. L., Phizackerley R. P. and Saul F. (1973) Three-dimensional structure of the Fab' fragment of a human immunoglobulin at 2.8-Å resolution. *Proc. natn. Acad. Sci. U.S.A.* **70**, 3305–3310.

Poljak R. J., Amzel L. M., Chen B. L., Phizackerley R. P. and Saul F. (1974) The three-dimensional structure of the Fab' fragment of a human myeloma immunoglobulin at 2.0-Å resolution. *Proc. natn. Acad. Sci. U.S.A.* **71**, 3440–3444.

Potter M., Padlan E. A. and Rudikoff S. (1976) Localized insertion–deletion mutations: a major factor in the evolution of immunoglobulin structural variability. *J. Immunol.* **117**, 626–629.

Prasad L., Vandonselaar M., Lee J. S. and Delbaere L. T. J. (1988) Structure determination of a monoclonal Fab fragment specific for histidine-containing protein of the phosphoenolpyruvate:sugar phosphotransferase system of *Escherichia coli*. *J. biol. Chem.* **263**, 2571–2574.

Pumphrey R. (1986) Computer models of the human immunoglobulins: shape and segmental flexibility. *Immunol. Today* **7**, 174–178.

Queen C., Schneider W. P., Selick H. E., Payne P. W., Landolfi N. F., Duncan J. F., Avdalovic N. M., Levitt M., Junghans R. P. and Waldmann T. A. (1989) A humanized antibody that binds to the interleukin 2 receptor. *Proc. natn. Acad. Sci. U.S.A.* **86**, 10,029–10,033.

Rajan S. S., Ely K. R., Abola E. E., Wood M. K., Colman P. M., Athay R. J. and Edmundson A. B. (1983) Three-dimensional structure of the Mcg IgG1 immunoglobulin. *Molec. Immun.* **20**, 787–799.

Riechmann L., Clark M., Waldmann H. and Winter G. (1988) Reshaping human antibodies for therapy. *Nature* **332**, 323–327.

Rini J. M., Schulze-Gahmen U. and Wilson I. A. (1992) Structural evidence for induced fit as a mechanism for antibody–antigen recognition. *Science* **255**, 959–965.

Sarma R. and Laudin A. G. (1982) The three-dimensional structure of a human IgG1 immunoglobulin at 4 Å resolution: a computer fit of various structural domains on the electron density map. *J. appl. Cryst.* **15**, 476–481.

Sarma V. R., Silverton E. W., Davies D. R. and Terry W. D. (1971) The three-dimensional structure at 6 Å resolution of a human γG1 immunoglobulin molecule. *J. biol. Chem.* **246**, 3753–3759.

Sasisekharan V. (1962) Stereochemical criteria for polypeptide and protein structures. In *Collagen* (Edited by Ramanathan N.), pp. 39–77. John Wiley & Sons, New York.

Satow Y., Cohen G. H., Padlan E. A. and Davies D. R. (1986) Phosphocholine binding immunoglobulin Fab McPC603. An X-ray diffraction study at 2.7 Å. *J. molec Biol.* **190**, 593–604.

Saul F. A. and Poljak R. J. (1992) Crystal structure of human immunoglobulin fragment Fab New refined at 2.0 Å resolution. *PROTEINS: Struct. Funct. Genet.* **14**, 363–371.

Schiffer M., Ainsworth C., Xu Z.-B., Carperos W., Olsen K. A., Solomon A., Stevens F. J. and Chang C.-H. (1989) Structure of a second crystal form of Bence–Jones protein Loc: strikingly different domain associations in two crystal forms of a single protein. *Biochemistry* **28**, 4066–4072.

Schiffer M., Chang C.-H., Naik V. M. and Stevens F. J. (1988) Analysis of immunoglobulin domain interactions. Evidence for a dominant role of salt bridges. *J. molec. Biol.* **203**, 799–802.

Schiffer M., Girling R. L., Ely K. R. and Edmundson A. B. (1973) Structure of a λ-type Bence–Jones protein at 3.5-Å resolution. *Biochemistry* **12**, 4620–4631.

Schlom J. (1991) Antibodies in cancer therapy: basic principles and applications. In *Biologic Therapy of Cancer: Principles and Practice* (Edited by DeVita Jr V. T., Hellman S. and Rosenberg S. A.), pp. 464–481. J. B. Lippincott, Philadelphia.

Schlom J., Milenic D. E., Roselli M., Colcher D., Bird R., Johnson S., Hardman K. D., Guadagni F. and Greiner J. W. (1991) New concepts in monoclonal antibody based radioimmunodiagnosis and radioimmunotherapy of carcinoma. *Int. J. Rad. appl. Instrum.* **18**, 425–435.

Schulz P. (1988) The interplay between chemistry and biology in the design of enzymatic catalysts. *Science* **240**, 426–433.

Segal D. M., Padlan E. A., Cohen G. H., Rudikoff S., Potter M. and Davies D. R. (1974) The three-dimensional structure of a phosphorylcholine-binding mouse immunoglobulin Fab and the nature of the antigen binding site. *Proc. natn. Acad. Sci. U.S.A.* **71**, 4298–4302.

Sheriff S., Silverton E. W., Padlan E. A., Cohen G. H., Smith-Gill S. J., Finzel B. C. and Davies D. R. (1987) Three-dimensional structure of an antibody–antigen complex. *Proc. natn. Acad. Sci. U.S.A.* **84**, 8075–8079.

Shoham M., Proctor P., Hughes D. and Baldwin E. T. (1991) Crystal parameters and molecular replacement of an anti-cholera toxin peptide complex. *PROTEINS: Struct. Funct. Genet.* **11**, 218–222.

Silverton E. W., Navia M. A. and Davies D. R. (1977) Three-dimensional structure of an intact human immunoglobulin. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5140–5144.

Stanfield R. L., Fieser T. M., Lerner R. A. and Wilson I. A. (1990) Crystal structures of an antibody to a peptide and its complex with peptide antigen at 2.8 Å. *Science* **248**, 712–719.

Steiner L. A. (1985) Immunoglobulin disulfide bridges: theme and variations. *Biosci. Rep.* **5**, 973–989.

Steinman L. (1990) The use of monoclonal antibodies for treatment of autoimmune disease. *J. Clin. Immunol.* **10**, 30S–38S.

Steipe B., Plueckthun A. and Huber R. (1992) Refined crystal structure of a recombinant immunoglobulin domain and a complementarity-determining region 1-grafted mutant. *J. molec. Biol.* **225**, 739–753.

Stevens F. J., Westholm F. A., Panagiotopoulos N., Schiffer M., Popp R. A. and Solomon A. (1981) Characterization and preliminary crystallographic data on the V_L related fragment of the human κI Bence Jones protein Wat. *J. molec. Biol.* **147**, 185–193.

Strong R. K., Campbell R., Rose D. R., Petsko G. A., Sharon J. and Margolies M. N. (1991) Three-dimensional structure of murine anti-*p*-azophenylarsonate Fab 36-71. I. X-ray crystallography, site-directed mutagenesis, and modeling of the complex with hapten. *Biochemistry* **30**, 3739–3748.

Suh S. W., Bhat T. N., Navia M. A., Cohen G. H., Rao D. N., Rudikoff S. and Davies D. R. (1986) The galactan-binding immunoglobulin Fab J539: an X-ray diffraction study at 2.6 Å resolution. *PROTEINS: Struct. Funct. Genet.* **1**, 74–80.

Sutton B. J. and Phillips D. C. (1983) The three-dimensional structure of the carbohydrate within the Fc fragment of immunoglobulin G. *Biochem. Soc. Trans.* **11**, 130–132.

Tan L. K., Shopes R. J., Oi V. T. and Morrison S. L. (1990) Influence of the hinge region on the complement activation, C1q binding and segmental flexibility in chimeric human immunoglobulins. *Proc. natn. Acad. Sci. U.S.A.* **87**, 162–166.

Tempest P. R., Bremner P., Lambert M., Taylor G., Furze J. M., Carr F. J. and Harris W. J. (1991) Reshaping a human monoclonal antibody to inhibit human respiratory syncytial virus infection *in vivo Bio/Technology* **9**, 266–271.

Theriault T. P., Leahy D. J., Levitt M., McConnell H. M. and Rule G. S. (1991) Structural and kinetic studies of the Fab fragment of a monoclonal anti-spin label antibody by nuclear magnetic resonance. *J. molec. Biol.* **221**, 257–270.

Tormo J., Stadler E., Skern T., Auer H., Kanzler O., Betzel C., Blass D. and Fita I. (1992) Three-dimensional structure of the Fab fragment of a neutralizing antibody to human rhinovirus serotype 2. *Protein Sci.* **1**, 1154–1162.

Tramontano A., Chothia C. and Lesk A. M. (1990) Framework residue 71 is a major determinant of the position and conformation of the second hypervariable region in the V_H domains of immunoglobulins. *J. molec. Biol.* **215**, 175–182.

Tulip W. R., Varghese J. N., Laver W. G., Webster R. G. and Colman P. M. (1992*a*) Refined crystal structure of the influenza virus N9 neuraminidase–NC41 Fab complex. *J. molec. Biol.* **227**, 122–148.

Tulip W. R., Varghese J. N., Webster R. G., Air G. M., Laver W. G. and Colman P. M. (1989) Crystal structures of neuraminidase-antibody complexes. *Cold Spring Harbor Symp. Quant. Biol.* **54**, 257–263.

Tulip W. R., Varghese J. N., Webster R. G., Laver W. G. and Colman P. M. (1992*b*) Crystal structures of two mutant neuraminidase–antibody complexes with amino acid substitutions in the interface. *J. molec. Biol.* **227**, 149–159.

Verhoeyen M., Milstein C. and Winter G. (1988) Reshaping human antibodies: grafting an antilysozyme activity. *Science* **239**, 1534–1536.

Vitali J., Young W. W., Schatz V. B., Sobottka S. E. and Kretsinger R. H. (1987) Crystal structure of an anti-Lewis *a* Fab determined by molecular replacement methods. *J. molec. Biol.* **198**, 351–355.

Vix O., Rees B., Thierry J.-C. and Altschuh D. (1993) Crystallographic analysis of the interaction between cyclosporin A and the Fab fragment of a monoclonal antibody. *PROTEINS: Struct. Funct. Genet.* **15**, 339–348.

Waldmann T. A. (1991) Monoclonal antibodies in diagnosis and therapy. *Science* **252**, 1657–1662.

Wilson I. A., Stanfield R. L., Rini J. M., Arevalo J. H., Schulze-Gahmen U., Fremont D. H. and Stura E. (1991) Structural aspects of antibodies and antibody-antigen complexes. In *Catalytic Antibodies, Ciba Foundation Symp.* **159**, 13–39.

Winter G. P. (1989) Antibody engineering. *Phil. Trans. R. Soc. Lond. (Biol.)* **324**, 537–546.

Wu T. T. and Kabat E. A. (1970) An analysis of the sequences of the variable regions of Bence–Jones proteins and myeloma light chains and their implication for antibody complementarity. *J. exp. Med.* **132**, 211–249.

Wu T. T., Johnson G. and Kabat E. A. (1993) Length distribution of CDRH3 in antibodies. *PROTEINS: Struct. Funct. Genet.* **16**, 1–7.

Zheng Y., Shopes B., Holowka D. and Baird B. (1991) Conformation of IgE bound to receptor and in solution. *Biochemistry* **30**, 9125–9132.

Zheng Y., Shopes B., Holowka D. and Baird B. (1992) Dynamic conformations compared for IgE and IgG1 in solution and bound to receptors. *Biochemistry* **31**, 7446–7456.

FUNDAMENTAL IMMUNOLOGY

THIRD EDITION

Editor

WILLIAM E. PAUL, M.D.

Laboratory of Immunology
National Institute of Allergy and
Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Raven Press & New York

Raven Press, Ltd., 1185 Avenue of the Americas, New York, New York 10036

Made in the United States of America

Library of Congress Cataloging-in-Publication Data

Fundamental immunology/editor, William E. Paul.—3rd ed.
p. cm.
Includes bibliographical references and index.
ISBN 0-7817-0022-1
1. Immunology. I. Paul, William E.
[DNLM: 1. Immunity. QW 504 F9804 1993]
QR181.F84 1993
.616.079—dc20.
DNLM/DLC
for Library of Congress 93-9718
CIP

9 8 7 6 5 4 3 2 1

TABLE 3. *Comparison of the percent identity between the human V_H families* [a,b]

		V_H1	V_H2		V_H3		V_H4		V_H5		V_H6
Family	Gene	51p1	MC1	M60	V_H26	V_H3005	71-2	4-21	251	32	15p1
V_H1	20p3	[82]	34	32	53	53	48	50	65	66	38
	51p1		34	32	57	58	48	48	65	66	41
V_H2	MC1			[92]	47	49	57	51	38	40	51
	M60				48	48	55	52	39	40	49
V_H3	V_H26					[80]	55	54	53	56	50
	V_H3005						55	51	55	56	51
V_H4	71-2							[86]	50	52	68
	4-21								50	54	61
V_H5	251									[92]	41
	32										43

[a] Note the boxed values on the diagonals illustrating the >80% identity between family members.
[b] Numbers represent degree of identity between germline-encoded V regions at the protein level.

ability is much more limited—restricted to occasional small insertions (31) and sequence variation at one position. This "junctional diversity" in CDR3 serves to boost the number of possible different sequences to well over the number of B cell clones produced in an individual's lifetime (2). However, the overall number of potential antibody sequences is higher still due to somatic hypermutation, which can alter residues throughout the variable region.

Fv Structure and Diversity in Three Dimensions

The vast sequence repertoire resulting from somatic recombination and hypermutation provides merely the theoretical "realm of possibility" for diversity. The true repertoire of binding specificities results from the folding of variable region polypeptides into immunoglobulin domains and subsequent assembly into unique, competent Fv modules. Fv structure therefore provides the

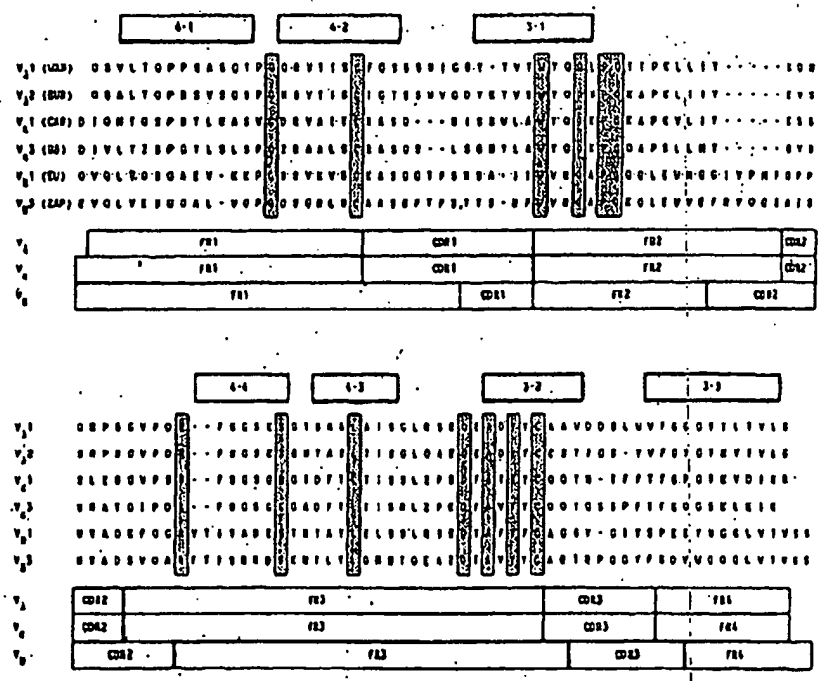


FIG. 9. Sequence alignments of six variable regions. Sequences are aligned using the DNASTAR® program (DNASTAR Inc., Madison, WI) to maximize homology by introducing gaps (–). Strongly conserved residues are *boxed* and *shaded*. *Boxes* below the sequences label the extent of the statistical features of V regions. Note the differences in length between heavy and light chain CDRs 1 and 2.

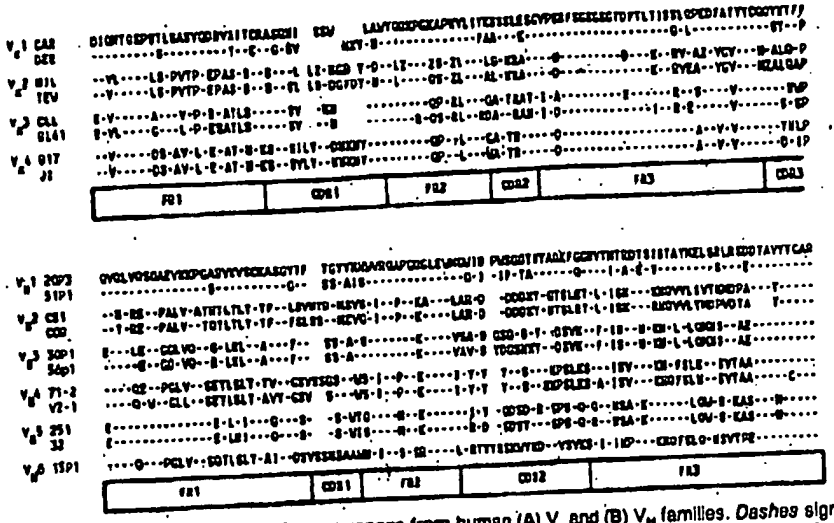


FIG. 10. Alignment of representative sequences from human (A) V_κ and (B) V_H families. *Dashes* signify identity with the top sequence while gaps are indicated with *blank spaces*. Again, boundaries of CDRs and FRs are labeled beneath the sequences. Note the substitutions common to each pair from a family relative to the top sequences ("linked substitutions").

three-dimensional context in which the different amino acids interact to create diverse ligand-binding sites (paratopes). As mentioned earlier, V domains possess solvent-exposed bends/loops that connect the strands of the β-barrel; the loops that fall within CDRs (termed H1, 2, 3 and L1, 2, 3 in the heavy and light chain, respectively) are brought together by VH:VL dimerization to create the ligand-binding surface as in Fig. 8. Sequence variation of two types in these loops clearly affects ligand recognition and provides the most obvious source of structural variety. First, CDRs vary significantly in length, largely as function of germline V-gene usage; this provides considerable paratope plasticity in terms of overall surface area and number of adoptable conformations. Second, by definition CDRs vary in sequence composition based on gene usage, junctional diversity (CDR3), and hypermutation; this serves to alter local secondary structure and paratope surface properties such as charge, polarity, and aromaticity. Since framework residues near the CDR boundaries often directly interact with antigen (32), alterations in these amino acids similarly affect the paratope surface.

The hypervariable regions also display a critical and far more subtle form of diversity in the form of conformational variability. Of course CDR backbone trajectory is strongly influenced by alterations of resident amino acids, especially those (usually Pro and Gly) that allow bending through the adoption of unusual torsion angles (27); occasional N-glycosylation of CDR asparagine residues has also been implicated in alteration of loop conformation and binding properties (33,34). Additionally, however, CDR loops can extensively interact with each other and with nearby framework residues as shown by two particularly striking examples from x-ray crystallography. In the first, the heavy chain framework residue at position 71 was shown to determine the conformation of the short HCDR2 loop (35). In the second, an anti-azophenylarsonate antibody, HCDR1 served as the surface on which the long HCDR2 packed; also, a Tyr to Phe mutation in LCDR1 altered the conformation of HCDR3 (17). The hypothesis that binding site structure results from multiple interactions between CDRs and frameworks gains further support from efforts to "humanize" antibodies. In these experiments, the antigen-binding loops from rodent monoclonal antibodies are grafted into human Fv frameworks in an effort to produce nonantigenic immunoglobulins for human therapy (36). In one experiment, the loops from mAb D1.3 (anti-lysozyme K_D = 3.7 nM) were grafted into human frameworks that differed from the natural mouse frameworks by 48 amino acids—the total for heavy and light chains (37). The "humanization" was accompanied by a large drop in affinity to a K_D of 260 nM. Significantly, the reversion of one framework residue to wild type increased the k_{on} and reconstituted affinity to K_D = 77 nM; simultaneous reversion of three other framework residues decreased the k_{off} and further restored affinity to K_D = 14 nM.

The VH:VL dimer interface, of which 25% is CDR side chains (26), also influences paratope morphology. The two V domains dimerize via their five-strand sides in a manner unique among all known protein structures (26). The actual interface consists of the four strands C'-C-F-G. Usually β-sheets pack so that residues in the mid-

dle strands form most of the contacts between the layers; this is how the two sides of a domain sandwich associate and how constant domains dimerize. In V regions, however, conserved β-bulges in the edge strands (C' and G) flip the side chains from those strands into the interior of the dimer such that edge residues form the bulk of a cylindrical core as in Fig. 11. Due to this peculiar arrangement, alterations in core side chains (both CDR and FR) may result in rotations of the V domains of up to 6 degrees relative to each other (38)—resulting in slight repositioning of the CDRs and yet another means of structural diversification. As will be seen in Chapter 12, high affinity and specificity depend on a large number of precisely coordinated, weak interactions between the epitope and paratope. The multiple modes of three-dimensional diversity provide antibodies with the "fine-

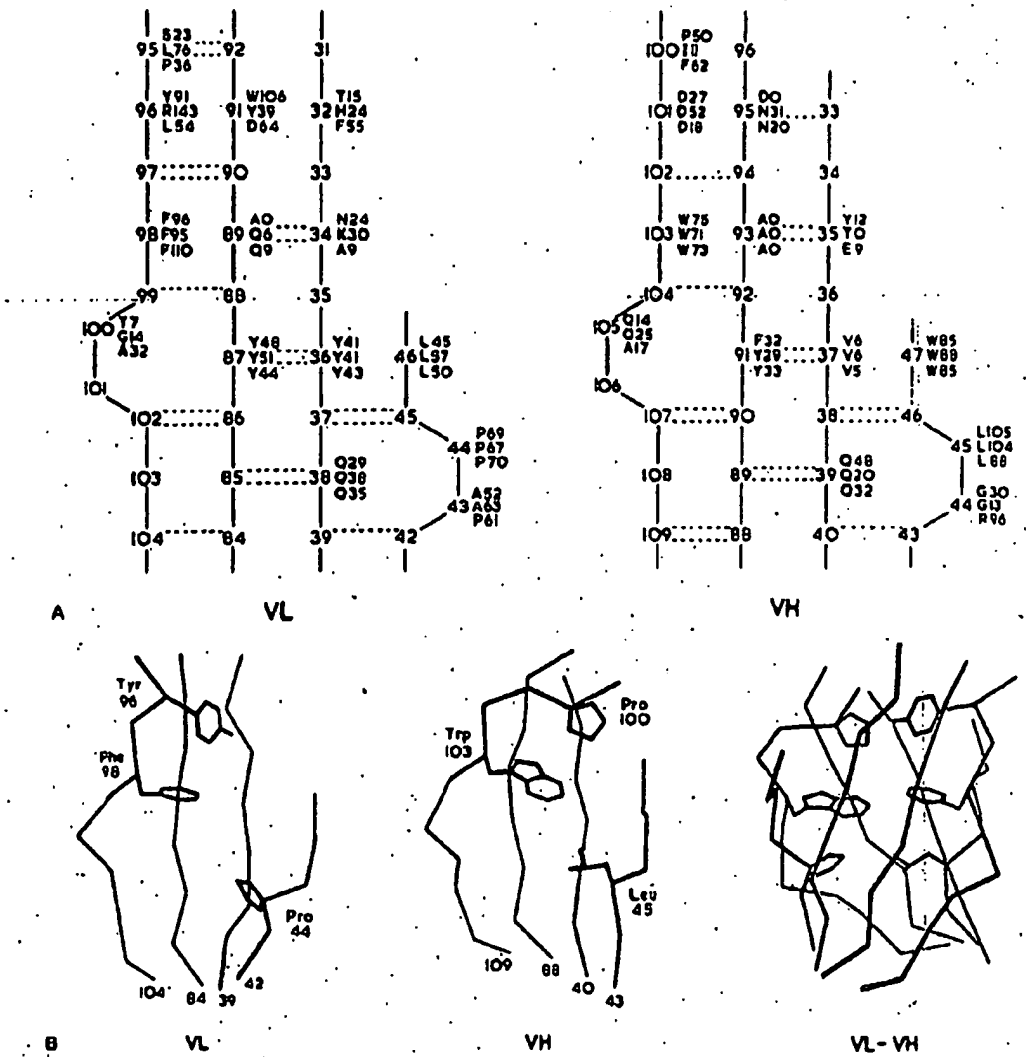


FIG. 11. The bulge structures in V regions. A: Two-dimensional view of V-region sequences along the dimerization face (four out of five strands are used). Consecutive residues are connected by *solid lines* while hydrogen bonds between β-strands are denoted by *dotted lines*. The conserved bulge residues are represented by protrusions on the edge strands C' and G. B: Three-dimensional representation of part A showing the flipping of edge residues into the interface. The right-most drawing shows the V_L and V_H faces associated to form the dimer interface. (From ref. 28, with permission.)

tuning" vital to the orchestration of those weak interactions.

Antigen–Antibody Interactions

As with all molecular associations, an antigen–antibody interaction occurs only if the binding reaction releases enough free energy to be thermodynamically favored—affinity correlating exponentially with free energy (covered in detail in Chapter 12). The required free energy is a sum of entropic and enthalpic components with increases in entropy and decreases in enthalpy favoring association. Few association reactions both increase entropy and decrease enthalpy, however; rather, a favorable change in one of the components overwhelms a smaller, unfavorable change in the other. For example, protein folding requires that much of the polypeptide become sequestered in hydrophobic core regions, which eliminates interactions between water molecules and much of the protein. The resultant gain in enthalpy presents a barrier to the folding process (as does the loss of protein conformational entropy following adoption of an ordered, native state). However, since the large numbers of excluded water molecules no longer are spatially constrained by interaction with the hydrophobic core, a large increase in solvent entropy takes place. It is this entropy gain that drives protein folding in the face of an enthalpic barrier. Antigen–antibody interactions exhibit the opposite relationship between entropy and enthalpy. As an immunoglobulin binds its ligand, the ability of one molecule to move relative to the other is lost; similarly, the binding reaction freezes most of the conformational motions of the epitope and the backbones and side chains of the paratope. Thus antibodies face significant entropic barriers to antigen binding.

The structural properties of antigen–antibody interactions directly reflect the need to overcome unfavorable entropy changes by both limiting the loss of entropy and maximizing the loss in enthalpy. At the amino acid level, this results in an enrichment for amino acids such as Tyr, Trp, Ser, and Asn in combining sites (39) (Table 4). These amino acids have fewer conformational degrees of freedom than the other amino acids and therefore less entropy to lose upon ligand binding. They additionally participate in a large variety of interactions such as hydrogen bonds, van der Waals, dipole–dipole, and aromatic π-stacking (Tyr and Trp); all of which contribute to the enthalpic changes required to drive the binding reaction. Of course other amino acids and other types of interactions (e.g., salt bridges) are employed as needed. The same thermodynamic considerations also dictate that the interacting surfaces of the antigen and the antibody be as large and as close as possible. Somewhat analogously to the protein folding situation, large interaction areas exclude more bound water—thus somewhat offsetting the loss of protein entropy with a gain in solvent entropy. Large contact areas, more importantly, contribute to binding enthalpy by allowing many simultaneous interactions of all sorts to take place between the epitope and paratope. Structurally, this need for maximum contact surface leads to two types of binding site: a deep cleft that snugly surrounds small ligands such as peptides and low molecular weight organics or a broad face that is complementary to an equally broad epitope on large protein antigens. Colorplates 1 and 2 show notable examples of each type. Colorplate 1 shows the complex between an eight amino acid peptide and its paratope—clearly demonstrating the intricate groove in which the antigen binds; fully 620 Å^2 of peptide are buried in the paratope—almost as much as in protein–antibody complexes (40). This much contact is required to overcome the extreme conformational flexibility that small peptides possess. Colorplate 1B also demonstrates a phenomenon typical of "small-ligand" interactions; namely, one of the CDRs (L2: the irregular protrusion high and to the left in the picture) makes no contact with the bound peptide. Colorplate 2 shows the *en face* type of interaction for three anti-lysozyme antibodies that recognize different epitopes (32). These complexes utilize all six CDRs and involve an average paratope area of approximately 710 Å^2. Note the variety, complementarity, and

TABLE 4. *Percentage composition of amino acids in different parts of antibodies of different species compared with the vertebrate average*

Amino Acid	Vertebrate average	Variable regions	CDR Loops	Potential binding positions	Known binding positions
ala	7.2	6.4	7.1	3.5	1.1
arg	4.9	3.6	3.8	2.8	5.7
asn	4.1	2.6	7.4	11.2	6.8
asp	5.4	3.8	5.1	6.1	8.0
cys	2.5	2.0	0.1	0.2	0.0
gln	3.9	5.7	4.3	0.8	1.1
glu	6.8	3.4	2.4	2.4	3.4
gly	7.6	9.4	7.0	8.7	8.0
his	2.2	0.8	2.4	3.9	2.3
ile	5.0	4.0	3.4	3.4	1.1
leu	8.7	7.8	5.1	2.4	1.1
lys	6.5	4.4	4.1	2.8	0.0
met	2.3	1.8	2.1	0.2	0.0
phe	4.1	3.0	2.5	2.3	3.4
pro	4.9	4.5	2.8	3.7	3.4
ser	7.0	13.8	17.3	14.9	13.6
thr	5.6	8.7	6.1	5.9	4.6
trp	1.3	2.1	1.9	5.5	10.2
tyr	3.2	5.3	11.1	17.3	25.0
val	6.8	6.9	4.1	2.3	1.1

Adapted from ref 39.

The "known positions" column shows frequencies derived from six Fab–ligand structures obtained from crystallography. The other column headings present usage frequencies obtained from predictions based on the sequences compiled in ref. 23. Residues mentioned in the text are boxed.

0022-1767/93/1503-880$02.00/0
The Journal of Immunology

Vol 150, 880–887, No. 3, February 1, 1993
Printed in U.S.A.

Lack of Promiscuity in Autoantigen-Specific H and L Chain Combinations as Revealed by Human H and L Chain "Roulette"[1]

Stefano Portolano,[2]* Gregorio D. Chazenbalk,* J. Scott Hutchison,† Sandra M. McLachlan,* and Basil Rapoport*

*Thyroid Molecular Biology Unit, Veterans' Administration Medical Center and the University of California, San Francisco, CA 94121 and †Nichols Institute, San Juan Capistrano, CA 92690

ABSTRACT. Individual H or L chains from a human autoantibody were used to search for other L or H chains that could form antigen-binding fragments, Fab, with the same specificity. The parent Fab (SP1.2) exhibits high affinity binding for thyroid peroxidase (TPO), a 107-kDa protein that is the major autoantigen in human autoimmune thyroiditis. This autoantibody "roulette," performed by using Ig H and L chain gene libraries expressed in bacteria, increased the frequency of TPO-binding clones in the new libraries. However, the frequency was still much lower than would be the case if promiscuous combinations with a variety of H or L chains were compatible with specific Ag binding. Nucleotide sequence analysis of the H and L chains of the new TPO-binding clones revealed even more restriction. Thus, with the SP1.2 H chain, all 11 new Fab utilized L chains from the same $V_\kappa 1$ family germline gene as SP1.2 itself. Similarly, five of six H chains "captured" by the SP1.2 L chain were very closely related to the SP1.2 H chain. However, one totally different H chain was isolated: SP4.6 has a V_H region that differs substantially from that of SP1.2. SP4.6 also has a distinct D region, uses a different J_H, and, unlike SP1.2, which is an IgG1, belongs to subclass IgG4. The affinities for TPO of SP4.6 (with the different H chain) and SP1.20 (which had the least mutated L chain germline gene) were similar to that of SP1.2 ($\sim 10^{-10}$ M). As expected, the SP1.2 and SP1.20 Fab, which have the same H chain and closely related L chains, bound to the same domain on TPO. However, a similar domain on TPO was recognized by both SP4.6 and SP1.2, despite the fact that their V, D, and J regions are quite different. This observation raises the possibility that the L chain is critical in defining epitope specificity, even in the presence of completely different D regions and nonidentical V_H regions. *Journal of Immunology,* 1993, 150: 880.

A hallmark of autoimmune thyroid destruction in humans is the presence in serum of high affinity IgG class autoantibodies to TPO,[3] the primary enzyme involved in thyroid hormone synthesis (reviewed in Reference 1). TPO is a glycoprotein of~ 107 kDa expressed on the surface of thyroid cells (reviewed in Reference 1). Human autoantibodies to TPO are not monoclonal, as evidenced by the contribution of different IgG subclasses and L chain types in the same patient (2, 3).

We have cloned three human IgG1/κ autoantibodies (SP1.2, SP1.4, and SP1.5; previously called SP2, -4, and -5) that bind TPO specifically and with high affinity (4, 5). These autoantibodies were obtained by expressing random combinations of H and L chain Ig genes as Ag-binding fragments, Fab, in a bacteriophage λ library (6) (Fig. 1*A*). The cDNA in this library was prepared from B cells infiltrating the thyroid gland of a patient with autoimmune

Received for publication July 15, 1992. Accepted for publication November 3, 1992.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This reseach was supported by National Institutes of Health Grants DK-36182 and EY-09498.

[2] Address correspondence and reprint requests to Stefano Portolano, M.D., Veterans' Administration Medical Center, Thyroid Molecular Biology Unit (111T), 4150 Clement Street, San Francisco, CA 94121.

[3] Abbreviation used in this paper: TPO, thyroid peroxidase.

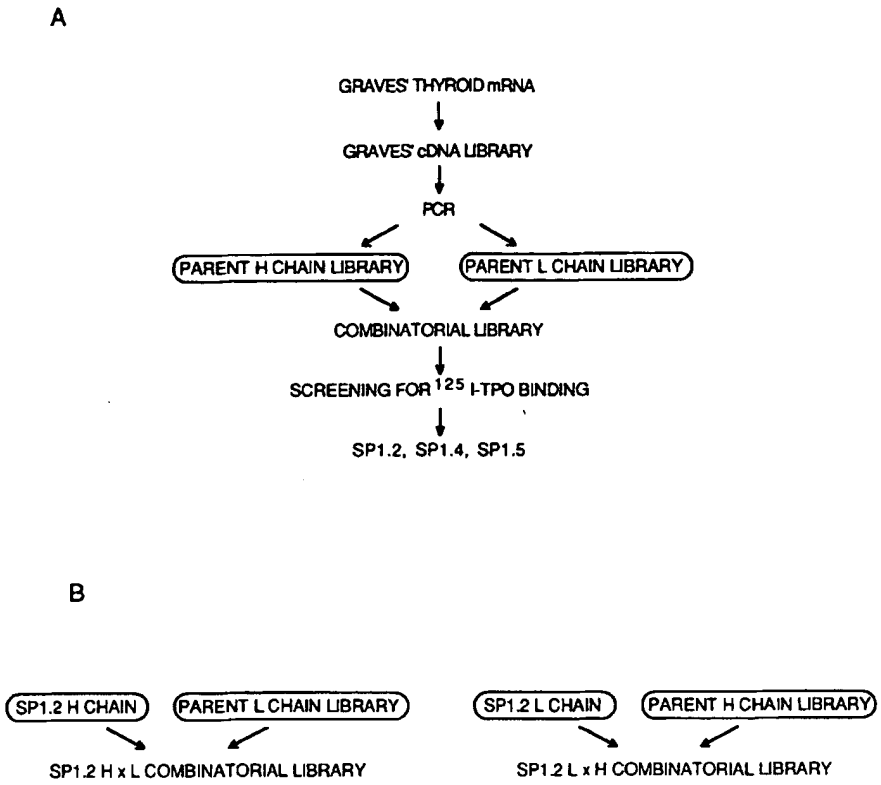


FIGURE 1. *A*, Outline of the approach (6) used to construct parent H and L chain libraries and the combinatorial library from which TPO-specific Fab SP1.2, SP1.4, and SP1.5 were obtained (4, 5). *B*, combinatorial libraries constructed by using SP1.2 H (or L) chain with the parent L (or H) chain. *PCR*, polymerase chain reaction.

thyroid disease. The three TPO autoantibody Fab share the identical Ig H chain. The L chains are not the same but are derived from the same V_κ germline gene, HSIGKLO12 (7).

Because of the random nature of the H and L chain combinations in our cDNA library, the question arose as to whether the SP Fab autoantibody H chain (or L chain) could combine with a variety of other L chains (or H chains) in the parent library and still bind to TPO. Therefore, we used the SP1.2 H (or L chain) to search for other L (or H) chains in the parent libraries that could form a Fab capable of binding TPO (Fig. 1*B*). Recently, following a similar approach, it has been reported that there is notable promiscuity in the H and L chain combinations of murine Fab that bind the hapten nitrophenyl phosphonamidate (8). In contrast, our "roulette" experiments demonstrate more restriction in the H and L chain combinations for high affinity binding to a human autoantigen, TPO.

Materials and Methods

Library construction and screening

The plasmid of clone SP1.2 (4) was digested with *Xho*I and *Spe*I to release the H chain cDNA insert. Similarly, the L chain cDNA fragment was obtained by *Xba*I and *Sac*I digestion. The inserts were gel purified and ligated into Immunozap H and L arms (Stratacyte, La Jolla, CA), respectively, to generate a clonal SP1.2 H chain library and a clonal SP1.2 L chain library, respectively (Fig. 1*B*). These libraries were amplified and the DNA was extracted as described (4). The DNA from the SP1.2 H chain library was digested with *Hind*III followed by *Eco*RI and was ligated with the DNA prepared from the original L chain library (4). Similarly, the DNA from the SP1.2 L chain library was digested with *Mlu*I followed by *Eco*RI and was ligated with the DNA from the original H chain library (4). The original H and L libraries contained >80% inserts of the correct size. The combinatorial libraries were screened in XL1-Blue cells by conventional techniques (9), by using secreted human rTPO (10) that had been labeled with ^{125}I (to a specific activity of 10 to 20 μCi/μg of protein) by the Iodogen method (11). TPO-binding plaques were cloned to homogeneity and plasmids were excised from the Immunozap bacteriophage by using the helper phage R408, according to the Stratacyte protocol. Nucleotide sequencing of the cDNA inserts was performed by the dideoxynucleotide chain termination method (12).

Fab expression

Fab were expressed as soluble proteins in XL1-Blue cells, as described previously (5). In brief, protein synthesis was induced with 1 mM isopropylthiogalactopyranoside (Sigma Chemical Co., St. Louis, MO) for 1 h at 37°C. The

Table I
Frequencies of ^{125}I-TPO-binding clones in roulette of SP1.2 H and L chains

Fab Combinatorial Library	Frequency	Plaques Screened
SP H chain × SP L chain	1:60,000	180,000
SP1.2 L chain × SP H chain	1:5,000	30,000
SP1.2 H chain × SP L chain	1:500	15,000

cells were then pelleted, frozen at −20°C, and resuspended in 0.02 volumes of 10 mM Tris, pH 8.0, containing 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.1 mM PMSF (all from Sigma). The suspension was sonicated, membranes were pelleted by centrifugation at 4000 × *g*, and the Fab were affinity purified from the supernatant by using a protein G-Sepharose column (Pharmacia, Piscataway, NJ).

Fab binding of ^{125}I-TPO

As described previously (5), Fab diluted in assay buffer (0.15 M NaCl containing 10 mM Tris·HCl, pH 7.5, and 0.5% BSA) were incubated with ^{125}I-TPO (~25,000 cpm) and mouse mAb to human κ L chains (QE11; Recognition Sciences, Birmingham, UK), in a total volume of 200 μl. After 1 h at room temperature, 100 μl of Sac-cel (donkey anti-mouse Ig covalently coupled to cellulose; IDS, Boldon, Tyne, and Wear, UK) were added, and the incubation was continued for 30 min. After addition of 1 ml of assay buffer and vortexing, the mixture was centrifuged for 5 min at 1000 × *g* to sediment the immune complexes, which were then counted to determine the percentage of radiolabeled TPO bound. The affinities of the Fab for TPO were determined by Scatchard analysis (13) from values obtained in the presence of increasing concentrations of unlabeled TPO. The data presented are the mean ± SEM of triplicate determinations.

Competition between Fab for binding to TPO

One Fab was immobilized by incubation (total volume, 200 μl) with murine mAb anti-human κ (QE11) for 1 h at room temperature. After incubation with 100 μl of Sac-cel

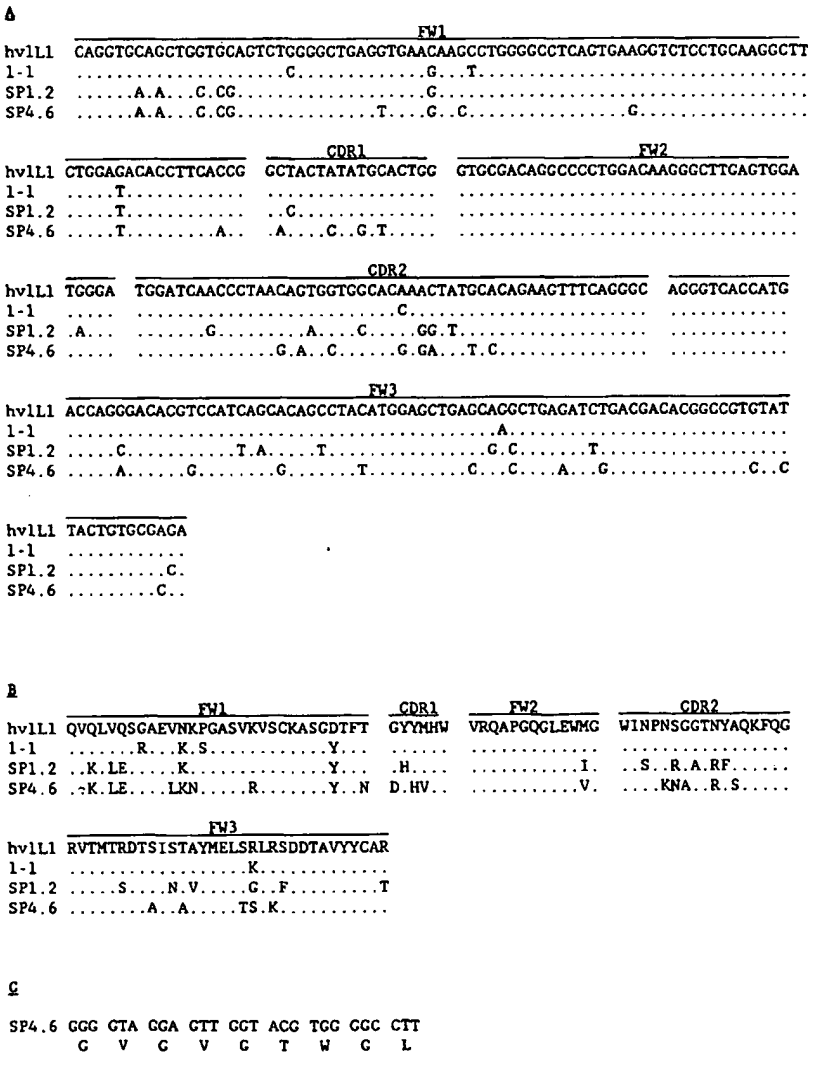


FIGURE 2. *A*, Nucleotide sequences of the V_H region of TPO-specific Fab SP1.2 and SP4.6, compared with germline genes hv1L1 (16) and 1–1 (14). *B*, derived amino acid sequence (in single-letter code) of the V_H regions of SP1.2 and SP4.6, again in comparison with hv1L1 and 1–1. In *A* and *B*, *dots* represent identical nucleotides or amino acids, respectively. CDR and framework regions (*FW*) are indicated according to Kabat et al. (26). *C*, nucleotide and derived amino acid sequences of the SP4.6 D region. These sequence data are available from GenBank (accession number M82813 for SP1.2) and from EMBL (accession number Z15084 for SP4.6).

(30 min at room temperature), the complexes were diluted in assay buffer (see above) and centrifuged at 1000 × *g* (5 min at 4°C). The pellets were resuspended in normal human serum diluted 1/30 in assay buffer, to saturate remaining anti-κ binding sites. In a separate set of tubes, increasing concentrations of "free" Fab were preincubated with ^{125}I-TPO for 1 h at room temperature. Aliquots (100 μl) were then incubated for 30 min with the immobilized Fab pellets and washed with assay buffer, and radioactivity bound to the Sac-cel was counted. Nonspecific binding (~2% of total counts added) was substracted to provide values for specific binding to TPO.

Results

Frequencies of ^{125}I-TPO-binding clones

Combining the L chain of SP1.2 with the SP parent H chain library yielded a combinatorial library of ~10^7 PFU (SP1.2 L × H library). Similarly, the combination of the SP1.2 H chain with the SP parent L chain library resulted in a library of ~10^7 PFU (SP1.2 H × L). As expected, this "roulette" with either the SP1.2 L or H chain led to a greater frequency of TPO-binding clones than was detected in the original screening of the parent library (Table I). Further, the frequency of TPO-binding clones was 10-fold greater in the SP1.2 H × L library than in the SP1.2 L × H library.

TPO-binding clones from the SP1.2 L × H library

We originally reported (4) that the V_H of SP1.2 appears to be derived from the V_H1 family germline gene 1–1 (14). V_H1–1 was first reported to be a pseudogene, but other investigators have suggested that 1–1 is a functional gene (15). More recently, another V_H1 germline gene, hv1L1, has been described (16) that is closely related to 1–1. At the nucleotide level, SP1.2 is more homologous to hv1L1 (93%) than to 1–1 (90%) (Fig. 2*A*).

Determination of the nucleotide sequences of the six new TPO-binding clones isolated from the SP1.2 L × H library revealed that the VDJ regions of five (SP1.7 to -11) are almost identical to those of the original SP1.2 (Table II). In the V_H region, three clones (SP1.9, SP1.10, and SP1.11) are identical to SP1.2 H chain and two clones (SP1.7 and SP1.8) differ from the original SP1.2 H chain by only one amino acid (proline and leucine for Ala-24, respectively). The D regions of SP1.7 to -11 are identical and differ from the SP1.2 D region by a single silent base substitution. The J regions of SP1.7 to -11 are of the J_H6 family (15). Re-examination of the SP1.2 DJ regions indicates that this clone, too, is a J_H6 (rather than a J_H3) combined with a very short D region.

One of the six new clones (SP4.6) from the SP1.2 L × H library differs more substantially from SP1.2 as well as from SP1.7 to -11 (Table II). Both SP4.6 and SP1.2 are members of the V_H1 family and may be derived from the germline gene hv1L1 (16) (Fig. 2*A*). However, there are major differences (24 amino acids) in the V_H regions of SP4.6 and SP1.2 (Fig. 2*B*). In addition, the SP4.6 D region (Fig. 2*C*) is quite different from that of SP1.2 and does not resemble any published D region sequence. Further, the SP4.6 J region is a J_H4 truncated at its 5′ end. The most surprising finding was that the hinge region sequence indicates that SP4.6 is an IgG4, in contrast to SP1.2 and SP1.7 to -11, which are IgG1. The C region primer used for the polymerase chain reaction in constructing the SP H chain parent library (CH1; Stratacyte, San Diego CA) is described as an IgG1 primer with the capacity to cross-prime with other IgG subclasses.

TABLE II
Characteristics at the nucleotide level of 17 TPO-binding clones in roulette of SP1.2 H and L chains[a]

SP1.2 L × H (6 clones)	V_H (%)	D	J_H	Subclass
SP1.7; -1.8	94	SP1.2	6	IgG1
SP1.9; -1.10; -1.11	94[b]	SP1.2	6	IgG1
SP4.6	90	Non-SP1.2	4	IgG4
SP1.2 H × L (11 clones)	**V_K (%)**		**J_K**	
SP1.17; -1.19	92[c]		2	
SP1.14; -1.15; -1.22	93[d]		2	
SP1.21	93[d]		?	
SP1.12	94		1	
SP1.13	93		2	
SP1.16	92		2	
SP1.18	93		2	
SP1.20	95		1	

[a] Homology of V_H and V_L genes is calculated for putative germline genes hvlL1 and HSIGKL012, respectively. The D regions are described as resembling SP1.2 (4) or not resembling SP1.2 (Fig. 2*C*).
[b] 100% homologous with SP1.2 H.
[c] 100% homologous with SP1.2 L.
[d] Identity of V_K for SP1.14, SP1.15, SP1.22, and SP1.21.

TPO-binding clones from the SP1.2 H × L library

The nucleotide sequences of the L chains were determined in 11 TPO-binding clones in the SP1.2 H × L library (Table II; Fig. 3). At the amino acid level, the V_κ regions of nine clones are very similar to, and two clones (SP1.17 and SP1.19) are identical to, the original SP1.2 L chain (Fig. 4*A*). Four of the nine clones (SP1.14, SP1.15, SP1.21, and SP1.22) have identical V_κ regions. All 11 new L chain clones appear to be derived from the V_κ germline gene HSIGKLO12 (7). This gene is almost identical to HUMIGKLVJ (17), with which we originally compared SP1.2 (4). SP1.20 is the closest (95%) to the putative germline gene (Table II). The J_κ regions of 10 of the 11 new clones belong to J_κ1 or J_κ2 (Fig. 4*B*). We cannot assign the J_κ region of SP1.21. SP1.14, SP1.15, and SP1.22 have identical V_κ and J_κ sequences. Because clones SP1.17 and SP1.19 have J_κ2 regions (like SP1.2) these clones are, overall, identical to SP1.2.

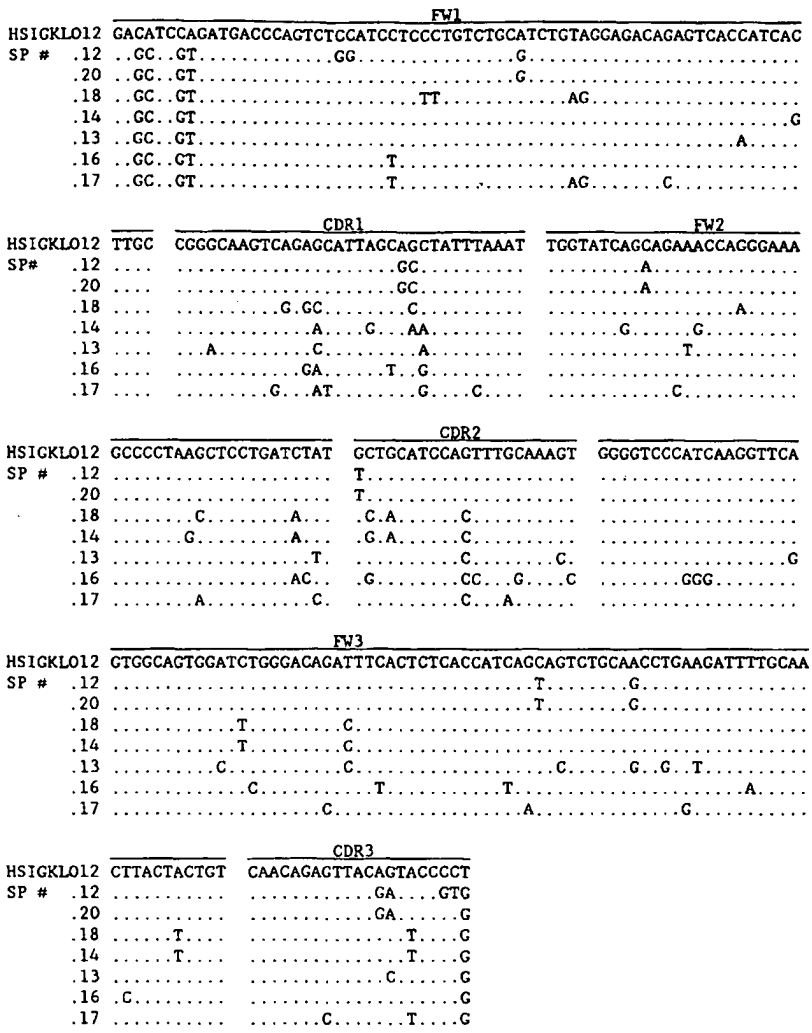


FIGURE 3. Nucleotide sequences of the V_κ regions of TPO-specific Fab SP1.12 through SP1.22, compared with the closest germline gene, HSIGKLO12 (7). The sequence of clone SP1.14 is identical to those of SP1.15, SP1.21, and SP1.22. Clone SP1.17 is identical to SP1.19 and both have the same sequence as SP1.2. *Dots*, identical nucleotides. CDR and framework regions (*FW*) are indicated according to Kabat et al. (26). These sequence data are available from EMBL under accession numbers Z15073 to Z15083 (SP1.12 to SP1.22).

Affinities for TPO of selected Fab

We expressed and purified the proteins for two of the newly isolated Fab clones, SP4.6 and SP1.20. SP4.6 was selected because of its distinctive H chain and SP1.20 because its L chain was the least mutated from the putative germline gene. Purified SP1.2 was already available (5). The affinities for TPO of the SP1.20 and SP4.6 Fab, calculated by Scatchard analysis (13) (Fig. 5), are very similar to each other (K_d, $1.0 \pm 0.2 \times 10^{-10}$ M and $1.4 \pm 0.3 \times 10^{-10}$ M, respectively) and to that of SP1.2 ($0.8 \pm 0.1 \times 10^{-10}$ M) (means ± SEM).

Binding domains on TPO for SP Fab

To determine whether there was overlap in the domains on TPO recognized by SP4.6, SP1.20, and SP1.2, we performed studies with an immobilized SP1.2 Fab. As expected, preincubation of ^{125}I-TPO with increasing concentrations of free SP1.2 inhibited the subsequent binding of the Ag to the immobilized SP1.2 (Fig. 6). The SP4.6 and SP1.20 Fab were equally effective, indicating that the binding domains of all three Fab overlapped. No competition was observed with another anti-TPO Fab, TR1.9, cloned from another patient.[4]

Discussion

In the present study, we chose a single H or L chain already known to confer high affinity ($\sim 10^{-10}$ M) specific binding for a large (107-kDa) protein autoantigen, TPO. We used this SP1.2 H (or SP1.2 L) chain to search for other L (or H) chains that could form a Fab capable of binding TPO. That is, we "spun the wheel" of the H and L chain repertoire of activated B cells infiltrating the patient's thyroid gland. As expected, the frequency of TPO-binding clones in the libraries generated by this biased recombination (1:500 for SP1.2 H × parent L chain and 1:5000 for SP1.2 L × parent H chain) was higher than in the original random combinatorial library (1:60,000) (4).

[4] Chazenbalk, G. D., Portolano, S., Russo, D., Hutchinson, J. S., Rapoport, B., McLachlan, S. "Human organ-specific autoimmune disease: molecular cloning and expression of an autoantibody gene repertoire." *Submitted for publication.*

A

		FW1	CDR1	FW2	CDR2
HSIGKLO12		DIVMTQSPSSLSASVGDRVTITC	RASQSISSYLN	WYQQKPGKAPKLLIY	AASSLQS
SP #	.12	EL.....G.....	.....A...	...K.....	S.....
	.20	EL.....	.....A...	...K.....	S.....
	.18	EL.....E.....	...RA..T...	.....R...N....	GT.T...
	.14	EL.....S.	....N.GK...	..R.....E....	GT.T...
	.13	EL.....	.T..T..R...	....I.....F	A.....T
	.16	EL.....	....D..R...	.....H	G..T.E.
	.17	EL.....E..T.....	...EN..R.S.	....Q.....S	...T...

		FW3	CDR3
HSIGKLO12		GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQSYSTP
SP #	.12	.....	....D..
	.20	.....	.....
	.18	.....F.....F.	.....S.
	.14	.....F.....F.	.....S.
	.13	.....G.....T...D.....	.....
	.16	...G.....Y.....	.....
	.17	.....H.....N....G.....	..T..S.

B

		CDR3	FW4
SP #	.12	GACACGTTC	GGCCACGGGACCAAGGTGGAAATCAAACGAACT
	.21	CCG..T...	....CT.....GA.....G.....
	.20	TGG.....	.....
	.18	T....T...	.....G.....C....G..AG.....
	.14	T....T...	.....G.....C....G..AG.....
	.13	T....T..T	.....G.....C....G.....G
	.16	TT...T..T	.....G.....C....G.....G.....G
	.17	TT...T..T	.....G.....C....G.....G

C

		CDR3	FW4	
SP #	.12	DTF	GHGTKVEIKRT	JK1
	.21	P..	.P..R..V...	JK?
	.20	W..	.....	JK1
	.18	Y..	.Q...L..E..	JK2
	.14	Y..	.Q...L..E..	JK2
	.13	Y..	.Q...L.....	JK2
	.16	F..	.Q...L.....	JK2
	.17	F..	.Q...L.....	JK2

FIGURE 4. *A*, Derived amino acid sequences of V_κ regions of anti-TPO Fab SP1.12 through SP1.22, compared with the closest germline gene, HSIGKLO12 (7). *B* and *C*, nucleotide (*B*) and derived amino acid (*C*) sequences of the J_κ regions of SP1.12 through SP1.22. *Dots*, identical nucleotides. CDR are indicated according to Kabat et al. (26).

However, the frequency was still lower (Table I) than would be the case if promiscuous binding to a variety of H or L chains was compatible with specific Ag binding.

The antibody repertoire in thyroid tissue B cells of patients with autoimmune thyroid disease is relevant to this discussion. This tissue is enriched, compared with draining lymph nodes and peripheral blood, in B cells actively secreting autoantibodies to the three major thyroid autoantigens, i.e., TPO, thyroglobulin, and the thyrotropin receptor (reviewed in Reference 18). This bias makes even more remarkable the paucity of H and L chains from this patient that are capable of combining with the preselected L or H chain to form a functional TPO binding site. For example, frequencies of 1:50 for a functional H chain have been reported in libraries from a mouse immunized with influenza hemagglutinin or in a human immunized with tetanus toxoid (8).

Nucleotide sequence analysis of the H and L chains of the new TPO-binding clones reveals even more restriction. Thus, with the SP1.2 H chain, all 11 new clones utilized L chains from the same $V_\kappa 1$ family germline gene. This germline gene was also used by the three other TPO Fab already described (4, 5). Amino acid substitutions predominate in the CDR regions. Overall, taking into account the V_κ and J_κ regions of the L chains of 11 new clones and the previously described SP1.2, SP1.4, and SP1.5 (5), only 10 were distinct, because several were identical to the SP1.2 L chain or to each other.

Additional evidence for restriction was the very limited variety of H chains "captured" by the SP1.2 L chain in generating new TPO-binding clones. Five of the six clones were very closely related to the SP1.2 H chain, which is also shared by the previously identified SP1.4 and SP1.5 clones (5). However, unlike the L chains, we did isolate one H chain, SP4.6, that differs from all others in the following respects: (i) there are 24 amino acid differences between the V_H regions of SP4.6 and SP1.2, although both may be derived from the same $V_H 1$ germline gene, hvlLl; (ii) the D region is totally distinct; (iii) SP4.6 uses a different J region; and (iv) SP4.6 belongs to the IgG4 subclass. The SP4.6 H chain is more rare in the parent

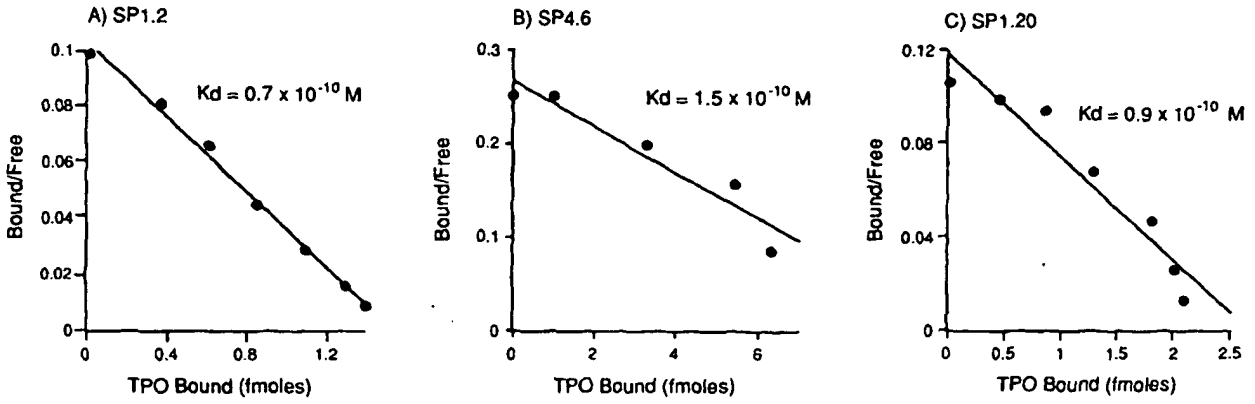


FIGURE 5. Scatchard analysis (see *Materials and Methods*) of the affinities of SP1.2, SP4.6, and SP1.20 for TPO. One of three representative experiments is shown for each Fab.

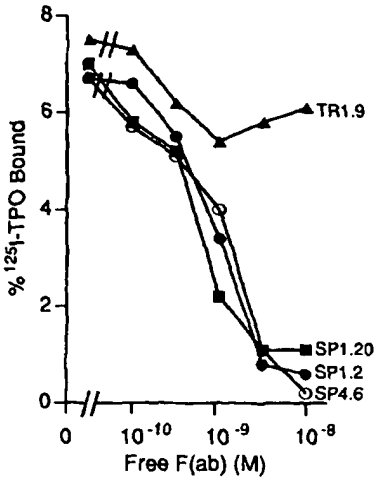


FIGURE 6. Binding domains on TPO for the SP1.2, SP4.6, and SP1.20 Fab. ^{125}I-TPO was preincubated in the absence or presence of increasing concentrations of SP4.6, SP1.20, and SP1.2 (*Free F(ab)*). Fab TR1.9 was obtained from another patient.[4] The ability of these complexes to bind to immobilized SP1.2 was then determined. The results are expressed as percentage of ^{125}I-TPO bound after subtraction of background values (~2%) obtained by using buffer alone.

library than is SP1.2 and related H chains, possibly because the oligonucleotide primer used to generate this library is suboptimal for IgG4. It is likely that the SP4.6 H chain was only found because of the bias introduced by the roulette approach.

In addition to providing data on the frequency and characterization of TPO-binding H and L chain combinations, our study illustrates important features with respect to antibody affinity and binding domains. These TPO autoantibodies have affinities for Ag several orders of magnitude greater than those reported for naturally occuring, polyreactive autoantibodies (K_d, 10^{-3} to 10^{-7} M) (19). It is possible that the screening approach that we used permits identification only of clones capable of high affinity TPO binding. Clones with lower affinity for TPO may, in the future, be identified more easily by using the phage display technique (20). However, this is not our present goal because TPO autoantibodies in patients' sera have high affinities (21), similar to our recombinant Fab.

It is of interest that the affinity of SP1.20 is high (similar to that of SP1.2) even though its V_κ chain is less mutated than that of SP1.2 (95% and 81% homology, respectively, to the germline gene). Assuming that both genes are, indeed, derived from HSIGKLO12, it is possible that affinity maturation of the L chain does not play a critical role in determining the affinity of the Fab for TPO.

The most unexpected finding in our study, in our estimation, concerns the TPO-binding domains of the SP Fab. Thus, in accordance with the data of Radic et al. (22) concerning murine autoantibodies to DNA, it was anticipated that both the SP1.2 and SP1.20 Fab, which have the same H chain and have closely related L chains, would interact with overlapping epitopes and bind to the same domain on TPO. However, a similar domain on TPO was recognized by both SP4.6 and SP1.2, despite the fact that their V, D, and J regions are quite different. This finding contrasts with the data of Martin et al. (23), who found that polyspecific autoantibody activity could only be generated with a specific D region. Our data were also unexpected in view of the extensive analysis by Kabat and Wu (24) of V region H and L chain combinations, which suggested V_H dominance in defining antibody specificity in many instances. However, as also suggested by Kabat and Wu (24) for some antibodies and as demonstrated by Smith-Gill et al. (25) for some murine antibodies to lysozyme, our findings raise the possibility that the L chain is critical in defining epitope specificity, even in the presence of completely different D regions and nonidentical V_H regions. With respect to our clones, it is possible that molecular modeling of SP4.6 and SP1.2 would reveal regions of similarity in their three-dimensional conformations, despite the dissimilarities in their CDR, particularly CDR3.

References

1. McLachlan, S. M., and B. Rapoport. 1992. The molecular biology of thyroid peroxidase: cloning, expression and role as autoantigen in autoimmune thyroid disease. *Endocr. Rev. 13:192.*
2. Parkes, A. B., S. M. McLachlan, P. Bird, and B. Rees Smith. 1984. The distribution of microsomal and thyroglobulin antibody activity among the IgG subclasses. *Clin. Exp. Immunol. 57:239.*
3. Weetman, A. P., C. M. Black, S. B. Cohen, R. Tomlinson, J. P. Banga, and C. B. Reimer. 1989. Affinity purification of IgG subclasses and the distribution of thyroid auto-antibody reactivity in Hashimoto's thyroiditis. *Scand. J. Immunol. 30:73.*
4. Portolano, S., P. Seto, G. D. Chazenbalk, Y. Nagayama, S. M. McLachlan, and B. Rapoport. 1991. A human Fab fragment specific for thyroid peroxidase generated by cloning thyroid lymphocyte-derived immunoglobulin genes in a bacteriophage *lambda* library. *Biochem. Biophys. Res. Commun. 179:372.*
5. Portolano, S., G. D. Chazenbalk, P. Seto, J. S. Hutchison, B. Rapoport, and S. M. McLachlan. 1992. Recognition by recombinant autoimmune thyroid disease-derived Fab fragments of a dominant conformational epitope on human thyroid peroxidase. *J. Clin. Invest. 90:720.*
6. Huse, W. D., L. Sastry, S. A. Iverson, A. S. Kang, M. Alting-Mees, D. R. Burton, S. J. Benkovic, and R. A. Lerner. 1989. Generation of a large combinatorial library of the immunoglobulin repertoire in phage *lambda*. *Science 246:1275.*
7. Pargent, W., A. Meindl, R. Thiebe, S. Mitzel, and H. G. Zachau. 1991. The human immunoglobulin κ locus: characterization of the duplicated O regions. *Eur. J. Immunol. 21:1821.*
8. Kang, A. S., T. M. Jones, and D. R. Burton. 1991. Antibody redesign by chain shuffling from random combinatorial immunoglobulin libraries. *Proc. Natl. Acad. Sci. USA 88:11120.*
9. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory, NY.
10. Foti, D., K. D. Kaufman, G. Chazenbalk, and B. Rapoport. 1990. Generation of a biologically-active, secreted form of human thyroid peroxidase by site-directed mutagenesis. *Mol. Endocrinol. 4:786.*
11. Salacinski, P. R. P., C. McLean, J. E. C. Sykes, V. V. Clement-Jones, and P. J. Lowry. 1981. Iodination of proteins, glycoproteins, and peptides using a solid-phase oxidizing agent, 1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril (Iodogen). *Anal. Biochem. 117:136.*
12. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA 74:5463.*
13. Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci. 51:660.*
14. Berman, J. E., S. J. Mellis, R. Pollock, C. L. Smith, H. Suh, B. Heinke, C. Kowal, U. Surti, L. Chess, C. R. Cantor, and F. W. Alt. 1988. Content and organization of the human Ig V_H locus: definition of three new V_H families and linkage to the Ig C_H locus. *EMBO J. 7:727.*
15. Pascual, V., and J. D. Capra. 1991. Human immunoglobulin heavy chain variable region genes: organization, polymorphism and expression. *Adv. Immunol. 49:1.*
16. Olee, T., E. W. Lu, D.-F. Huang, R. W. Soto-Gil, M. Deftos, F. Kozin, D. A. Carson, and P. P. Chen. 1992. Genetic analysis of self-associating immunoglobulin G rheumatoid factors from two rheumatoid synovia implicates an antigen-driven response. *J. Exp. Med. 1750:8310–842.*
17. Kato, S., K. Tachibana, N. Takayama, H. Kataoka, M. C. Yoshida, and T. Takano. 1991. Genetic recombination in a chromosomal translocation t(2;8)(p11;q24) of a Burkitt's lymphoma cell line, KOBK101. *Gene 97:239.*
18. Rees Smith, B., S. M. McLachlan, and J. Furmaniak. 1988. Autoantibodies to the thyrotropin receptor. *Endocr. Rev. 9:106.*
19. Nakamura, M., S. E. Burastero, Y. Ueki, J. W. Larrick, A. L. Notkins, and P. Casali. 1988. Probing the normal and autoimmune B cell repertoire with Epstein-Barr virus: frequency of B cells producing monoreactive high affinity autoantibodies in patients with Hashimoto's disease and systemic lupus erythematosus. *J. Immunol. 141:4165.*
20. Kang, A. S., C. F. Barbas, K. D. Janda, S. J. Benkovic, and R. A. Lerner. 1991. Linkage of recognition and replication functions by assembling combinatorial antibody Fab libraries along phage surfaces. *Proc. Natl. Acad. Sci. USA 88:4363.*
21. Beever, K., J. Bradbury, D. Phillips, S. M. McLachlan, C. Pegg, A. Goral, W. Overbeck, G. Feifel, and B. Rees Smith. 1989. Highly sensitive assays of autoantibodies to thyroglobulin and to thyroid peroxidase. *Clin. Chem. 35:1949.*
22. Radic, M. Z., M. A. Mascelli, J. Erikson, H. Shan, and M. Weigert. 1991. Ig H and L chain contributions to autoimmune specificities. *J. Immunol. 146:176.*
23. Martin, T., S. F. Duffy, D. A. Carson, and T. A. Kipps. 1992. Evidence for somatic selection of natural autoantibodies. *J. Exp. Med. 175:983.*
24. Kabat, E. A., and T. T. Wu. 1991. Identical V region amino acid sequences and segments of sequences in antibodies of different specificities. *J. Immunol. 147:1709.*
25. Smith-Gill, S. J., P. A. Hamel, T. B. Lovoie, and K. J. Dorrington. 1987. Contributions of immunoglobulin heavy and light chains to antibody specificity for lysozyme and two haptens. *J. Immunol. 139:4135.*
26. Kabat, E. A., T. T. Wu, H. M. Perry, K. S. Gottesman, and C. Foeller. 1991. *Sequences of Proteins of Immunological Interest.* United States Department of Health and Human Services, Bethesda MD.

Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 1979–1983, March 1982
Immunology

Single amino acid substitution altering antigen-binding specificity

(immunoglobulin/mutation/phosphocholine/antibody diversity)

STUART RUDIKOFF*, ANGELA M. GIUSTI†, WENDY D. COOK†, AND MATTHEW D. SCHARFF†

*Laboratory of Cell Biology, National Institutes of Health, Bethesda, Maryland; and †Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York

Communicated by Michael Potter, November 23, 1981

ABSTRACT **S107, a phosphocholine-binding myeloma protein, has been cloned in soft agar, and an antigen-binding variant has been isolated and characterized. The variant does not bind phosphocholine attached to carrier or as free hapten in solution but does retain antigenic determinants (idiotypes) of the parent. Chain recombination experiments suggest that the defect in binding is entirely in the heavy chain. Amino acid sequence analysis showed a single substitution—glutamic acid to alanine at position 35—in the first hypervariable or complementarity-determining region. In terms of the three-dimensional model of the phosphocholine-binding site, glutamic acid-35 provides a hydrogen bond to tyrosine-94 of the light chain that appears to be critical for stability of this portion of the binding site. The removal of this bond and the presence of the smaller alanine side chain is thus consistent with the loss in binding activity. These results suggest that small numbers of substitutions in antibodies, such as those presumably introduced by somatic mutation, may in *some* situations be effective in altering antigen-binding specificity.**

The generation of antibody diversity has long been and remains one of the intriguing questions in immunology. Protein sequence analyses (1, 2) and nucleic acid studies (3–7) have similarly suggested that the number of light (L) and heavy (H) chain genes in the germ line is large (>200 each). If random combinations of L and H chains were to occur, >10,000 different antibodies could be generated solely from the germ-line repertoire. Furthermore, immunoglobulin chains are encoded in multiple genetic elements. The variable domains of light chains are encoded by two gene segments designated variable (V) and joining (J) (8, 9). Heavy chain variable domains, in addition to V and J segments, have a third element, D (diversity), that encodes a portion of the third hypervariable region (10–12). The combination of a given L chain V gene with any of four functional J genes can thus produce additional structural diversity as can V, D, and J recombination in the H chain. The potential sequence diversity is further increased by variations in the sites at which these elements combine (8, 9, 13–17). At present, it is not clear how much the sequence diversity generated by these events contributes to functional changes that affect the specificity and affinity of antigen binding.

In view of the large amount of structural diversity that can be generated from the germ-line repertoire and the recombination events occurring during the formation of active immunoglobulin genes, the question of the occurrence and role of somatic mutation in the generation of antibody diversity remains to be determined. The initial studies of mouse λ L chains by Weigert and co-workers (18, 19) identified 12 invariant sequences, 5 with single amino acid substitutions, 1 with two substitutions, and 1 with three substitutions. All interchanges were located in hypervariable regions and it was concluded that the variants arose by a somatic mutation process. This interpretation has been confirmed by the finding that a single germ-line V region gene and J segment encode the λ_1 V regions (20). While the sequence changes in λ_1 L chains were confined to hypervariable regions, studies of a κ chain subgroup (V_{k21}) also suggested somatic mutations, but these substitutions were observed in framework as well as hypervariable residues (21). Most relevant to the studies described here is a recent report by Gearhart *et al.* (22) on the structure of H and L chains from phosphocholine (*P*-Cho)-binding hybridomas. A number of sequences were determined for IgM and IgG hybridomas and were compared with previously determined structures from IgA myeloma proteins. All of the μ chains were found to be identical in the V region, while all of the γ chains and about half of the α chains had substitutions that occurred in both hypervariable and framework regions. An analysis of the germ-line genes encoding the *P*-Cho heavy chains (23) suggested that all except one of the *P*-Cho heavy chains were encoded by a single gene that faithfully encodes the T15 IgA myeloma V region, as well as all of the μ V regions. These and similar findings among H chains from antinitrophenyl antibodies (24) argue that somatic mutation is occurring and that it is not restricted to hypervariable regions.

To date, it is not known whether the limited number of amino acid substitutions presumably generated by somatic mutation can be effective in altering antigen binding specificity or affinity. We have attempted to approach this question by examining the structure of antigen-binding variants derived from the *P*-Cho-binding myeloma protein S107. S107 is a BALB/c IgA, κ myeloma protein that appears to be identical in structure to the T15 myeloma protein and ≈90% of the BALB/c antibodies elicited by immunization with *P*-Cho-containing antigens (25). We have chosen this sytem because of the availability of a number of primary sequences from *P*-Cho-binding myeloma proteins and a three-dimensional structure of the Fab fragment from the closely related protein M603 (26, 27). Antigen-binding variants were selected by cloning S107 cells in soft agar and overlaying with *P*-Cho–keyhole limpet hemocyanin (KLH) (28). Clones that are not surrounded by antigen–antibody precipitates and occur at a spontaneous frequency of 0.1–1% are presumed to be antigen-binding variants. One of these first generation variants, U4, has been characterized in detail and is the subject of the present communication.

MATERIALS AND METHODS

The S107 cell line was originally obtained from the Salk cell bank. Propagation of the cells, their cloning, and identification

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: L and H, light and heavy chain genes or their respective polypeptide chains; V, J, and D, variable, joining, and diversity (region), respectively; *P*-Cho, phosphocholine; KLH, keyhole limpet hemocyanin; SRBC, sheep erythrocytes.

of variants have been described previously (28). *P*-Cho was attached to sheep erythrocytes (SRBC) (29), and hemagglutination of *P*-Cho–SRBC was determined as described by Evans *et al.* (30). Radioimmunoassays were carried out by the method of Pierce and Klinman (31) in which antigen (*P*-Cho–KLH) or antibody (28) was attached to polyvinyl plates (Dynatech). The S107 immunoglobulin and the rat anti-S107 monoclonal antibody were biosynthetically labeled by incubating the cultured myeloma cells with [^{35}S]methionine. Labeling of the antibodies and their use in the radioimmunoassay have been described (32). Equilibrium dialysis was carried out by the flow dialysis method of Colowick and Womack (33).

S107 and variant immunoglobulins were purified from the ascites of tumor-bearing animals. S107 was purified by affinity chromatography on *P*-Cho-Sepharose. The U4 protein was chromatographed on DEAE-Sephacel (Pharmacia) and then further purified by gel filtration on Sephacryl S200 (Pharmacia). Chain recombination was carried out as described by Manjula *et al.* (34) except that the denaturing buffer was 5 M guanidine in 0.2 M NH_4HCO_3.

For sequence studies, the H chains of both the S107 and U4 immunoglobulin were isolated on Sephadex G-100 columns equilibrated in 6 M urea/1 M HOAc. H chains were cleaved with CNBr and the fragments were isolated by gel filtration (35). Sequences were determined on a modified (36, 37) Beckman 890C sequencer using a Quadrol buffer system as described (38).

RESULTS AND DISCUSSION

Characterization of the U4 Variant. When a freshly isolated subclone of the S107 cell line is recloned in soft agar and overlayed with *P*-Cho–KLH, 0.1–1% of the clones are not surrounded by a visible antigen–antibody precipitate (28). Between 50% and 70% of these presumptive variant clones continue to secrete IgA molecules in the same amount as the parental clone. Most of these variant antibodies have normal-sized H and L chains and are polymeric molecules that are indistinguishable from the parental IgA (28, 39). One of these variants, U4, was grown to mass culture and injected into BALB/c mice, and the variant protein was purified from ascites fluid by DEAE-Sephacel and Sephacryl chromatography. Comparison of the antigen and hapten binding of the U4 variant and the parental S107 protein is presented in Table 1. U4 does not agglutinate *P*-Cho–SRBC and does not bind hapten when assayed by equilibrium dialysis. To further evaluate the antigen binding capacity of U4, *P*-Cho–KLH was absorbed to the walls of polyvinyl microtiter plates and unlabeled U4 and S107 protein were compared for their ability to compete with endogenously labeled S107 for antigen. U4 demonstrated minimal binding, only slightly more than an unrelated antibody, in competing for *P*-Cho in this assay. These results indicated that the U4 variant had lost its ability to bind antigen and hapten. When parent and U4 were analyzed for antigenic determinants (idiotypes) detected by a particular monoclonal antibody (Fig. 1),

Table 1. Antigen- and hapten-binding characteristics of myeloma S107 and variant U4

Cell line	Hemagglutination titer,* *P*-Cho–SRBC	Radioimmunoassay,† %*P*-Cho–KLH	K_a, 10^5 M^{-1}
S107.3.4	8192	100	2.3
U4	0	<0.01	0

* Purified protein (250 ng) was examined in 1:2 dilutions.
† % relative binding = (ng of parent required for 50% inhibition/ng of variant required for 50% inhibition) ×100.

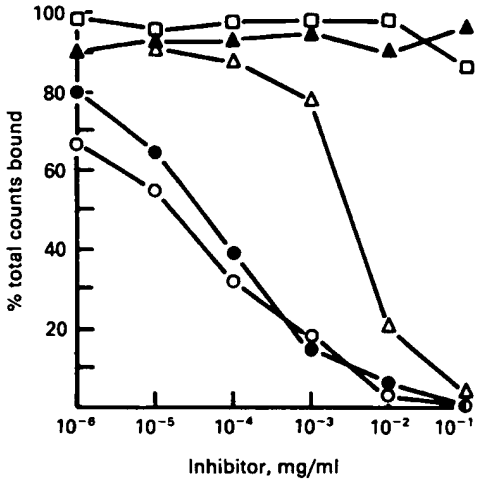


FIG. 1. Competition radioimmunoassay of S107 (●) and U4 (○) with a rat monoclonal antibody raised against S107. Purified S107 protein was attached to polyvinyl plates and parent and variant were incubated with 50% saturating amounts of [^{35}S]methionine-labeled rat monoclonal antibody in the presence of various amounts of the proteins indicated. M603 (▲) and M511 (△) are *P*-Cho-binding myeloma proteins. Reagents recognizing S107 frequently crossreact with M511. W3129 (□) does not bind *P*-Cho.

U4 was found to react with this reagent to approximately the same extent as the parent. By using conventional antisera to the *P*-Cho-binding site and a variety of additional antivariable region monoclonal antibodies, we have observed that some distinguish these two proteins to the extent that U4 reacts ≈1/10th as well as the parent while others fail to discriminate between parent and variant. Thus, the association of idiotype with a non-antigen-binding molecule (U4) is likely to be highly dependent on the particular reagent used. Taken together, the above data indicate that an alteration has occurred in the configuration of the U4 binding site that has drastically altered antigen-binding specificity but only minimally altered antigenic determinants.

To more precisely define the defect in U4, chain recombination experiments were performed between parent and variant. H and L chains were isolated from partially reduced proteins, mixed together in various combinations, and reassociated by removing the denaturing reagent (Table 2). The recombinants containing the H chain of U4 and the L chain of S107 did not hemagglutinate *P*-Cho–SRBC. However, the combination of the parental H chain and the U4 L chain not only agglutinated *P*-Cho–SRBC (Table 2) but also had the same affinity for *P*-Cho by equilibrium dialysis as the S107 protein (data not shown). The loss in antigen-binding activity was therefore associated entirely with the U4 heavy chain.

Structural Difference Between Parent and Variant. Since alterations in antigen binding were found to be associated with the U4 H chain, we have determined the primary structure of the entire H chain variable region of the variant and parental proteins. A single amino acid substitution—alanine for glutamic acid—was found at position 35 in the first hypervariable or complementarity-determining region (Fig. 2). This interchange can be explained by a single-base substitution in the second nucleotide position of the glutamic acid codon or could be due to a two-base change. A significant association between *P*-Cho binding and the occurrence of phenylalanine-32, tyrosine-33, and glutamic acid-35 has previously been noted (41). The effect of the observed alanine–glutamic acid interchange is striking in

Table 2. Chain recombination of S107 and U4

	Hemagglutination titer
$H^{107} + L^{107}$	10
$H^{107} + L^{U4}$	7.5
$H^{U4} + L^{107}$	1
$H^{U4} + L^{U4}$	1

Chain recombination was as described in *Materials and Methods*. The recombinants were not further purified. Equal amounts of protein, which also contained the same amount of idiotype by inhibition of hemagglutination, were allowed to react with *P*-Cho–SRBC. Facilitating rabbit anti-IgA antibody was added and the hemagglutinin titer is expressed as the square of the logarithm.

terms of the model of the *P*-Cho-binding site of M603 (Fig. 3). M603 is a *P*-Cho-binding myeloma protein whose three-dimensional structure has been determined (26). Crystallographic analysis has indicated that the binding of *P*-Cho hapten is almost entirely associated with amino acids in the H chain. When the H chains of M603 and S107 are compared, five differences are observed (four substitutions plus a one amino acid size difference), none of which occur at positions identified as participating in hapten contact. Although the L chains from M603 and S107 are from different V_κ subgroups and hence have considerable sequence variation (42–44), the one amino acid (leucine-96) directly implicated in hapten contact (26, 43) is also preserved in the S107 L chain. Furthermore, anti-*P*-Cho hybridomas have been identified (22) that are comprised of an M603 L chain paired with a T15 H chain and have affinities for *P*-Cho similar to that of the M603 myeloma protein (45).

Based on the above considerations as well as predictive calculations, we have concluded that the principles derived from the structure of the M603-binding site will largely be applicable to the S107 site (46). In the M603 structure, glutamic acid-35 was originally designated as a hapten-contacting residue (26). From continued refinement of the x-ray structure analysis, it now appears that the distance between *P*-Cho and glutamic acid-35 makes its role in direct hapten contact somewhat uncertain (D. R. Davies, personal communication). However, from Fig. 3, it can be seen that the side chain of glutamic acid-35 is hydrogen bonded to the phenolic hydroxyl of the L chain tyrosine-94 (43). This bond appears to be important in stabilizing this portion of the binding pocket that contains the hapten-contacting residues tyrosine-33 and arginine-52 from the H chain and leucine-96 from the L chain. In addition, the "vacuum" created by the removal of the carboxyl and methyl groups of glutamic acid would further distort this region. In fact, if glutamic acid-35 were only a hapten-contacting residue, it might be expected that substitution of the smaller alanine side chain would merely produce a decrease in affinity. This substitution, while possibly having only a minimal effect on hapten contact, apparently removes a bond and side chain essential to the structural integrity of this portion of the binding site. It is interesting to note that all H chains from conventionally induced anti-*P*-Cho antibodies are similar in amino acid sequence. From analysis of the gene family encoding the *P*-Cho H chain V regions (23), it can be seen that only one of three potentially expressed genes encodes glutamic acid at position 35. It is this gene that directly translates into the S107 protein sequence and presumably encodes most other *P*-Cho H chains. Thus, the predominant use of this particular gene in anti-*P*-Cho antibodies appears to correlate with the presence of glutamic acid-35. The two other genes code for serine at position 35 (as well as several additional differences), but the remaining major hapten-contacting residues are also present in these genes. One exception has been observed to date in that a single hybridoma (22) has been described that has a serine at position 35 and other substitutions characteristic of the two "unused" genes. This protein has the lowest affinity for *P*-Cho of any hybridoma or myeloma characterized thus far. It is thus possible that the presence of serine at residue 35 may maintain enough of the needed conformation to permit *P*-Cho binding (albeit at a lower affinity) in contrast to the complete loss associated with the alanine substitution in

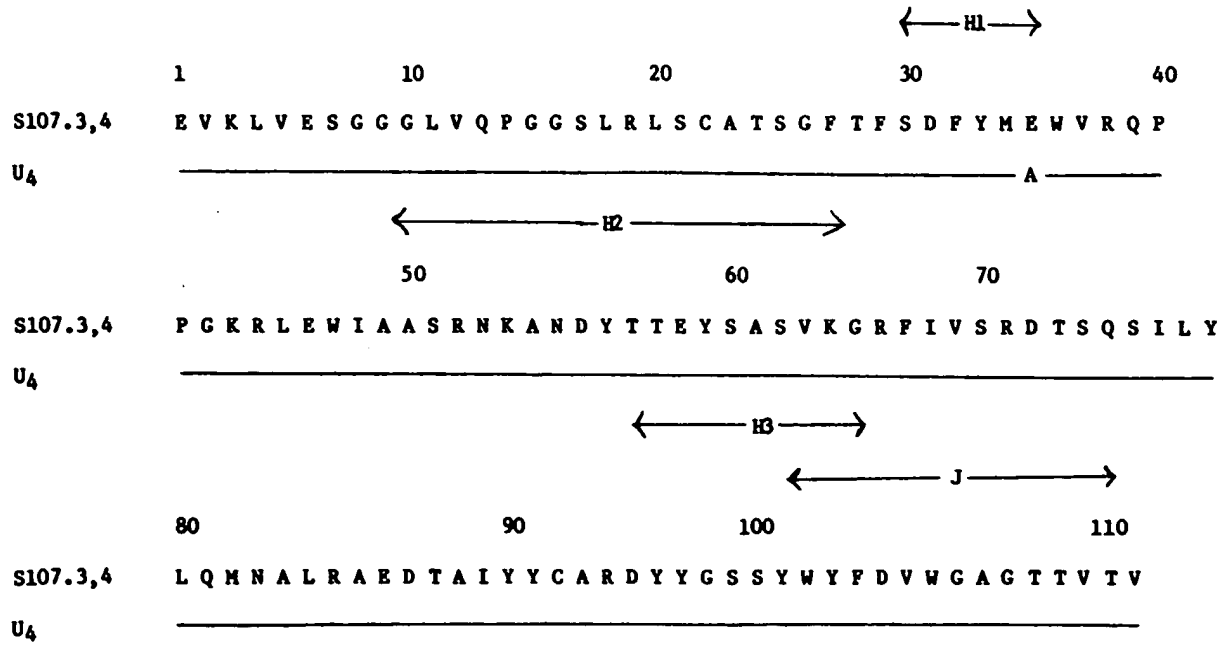


FIG. 2. Amino acid sequence of the H chain variable region of parent and variant. Cyanogen bromide fragments were isolated and their sequences were determined as described (35). Numbering is according to Kabat *et al.* (40).

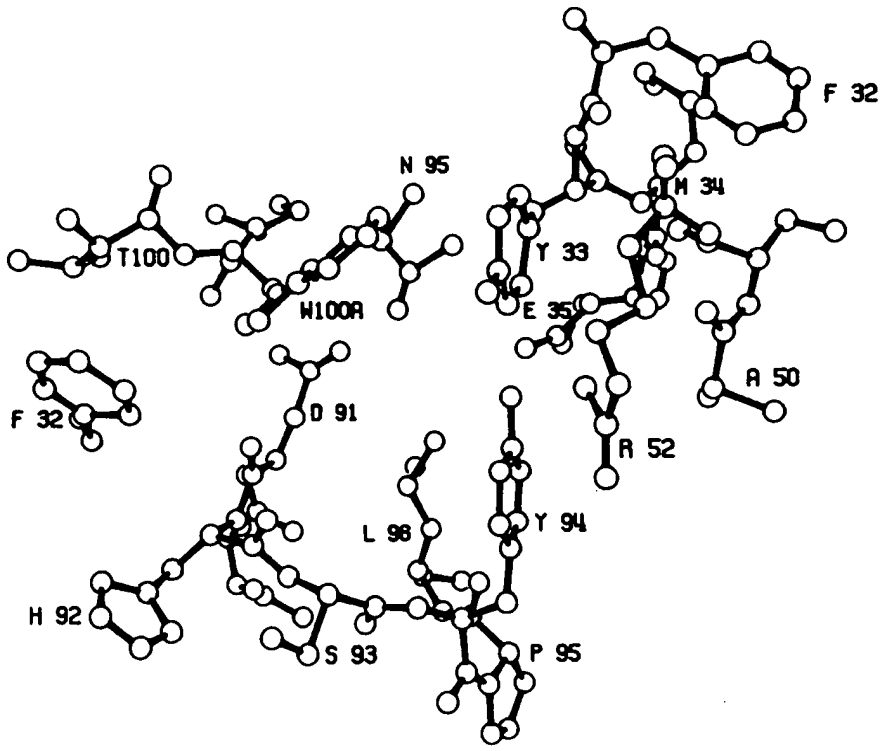


FIG. 3. Schematic representation of the *P*-Cho-binding site of M603 taken from ref. 43. H chain complementarity-determining regions are located in the upper portion. L chain residues contributing to the binding site occur mainly in the third L chain complementarity-determining region (lower portion). The effects of substitution in U4 are loss of the hydrogen bond between glutamic acid (E)-35 and tyrosine (Y)-94 and the accompanying decrease in side chain volume that appear to be critical to stability of this part of the site.

U4. Alternatively, the additional substitutions in this chain may partially compensate for the replacement of glutamic acid-35.

Although the H chains from *P*-Cho-binding antibodies are similar in amino acid sequence, L chains from three different V region groups that vary greatly in sequence may be associated with these H chains (42). However, all three of these L chains use the J5 joining segment and have the L chain sequence tyrosine-94, proline-95, leucine-96 (38, 43, 44), so that the bond between glutamic acid 35H and tyrosine 94L can be formed with any of these L chains. The sequence Tyr-Pro-Leu at positions 94, 95, and 96 is found only in L chains from *P*-Cho-binding proteins.

Implications for Generation of Diversity. We have shown that a single amino acid substitution is capable of completely altering antigen-binding specificity. Thus, a small number of amino acid substitutions, such as those postulated to arise by somatic mutation, can potentially be effective in generating antibody diversity in addition to that inherent in the germ-line repertoire. The high spontaneous frequency of mutants (28) observed for the generation of non-antigen-binding variants in our system further suggests that events of this nature are not infrequent. We have characterized another primary variant of S107 that has decreased antigen binding and a single amino acid substitution in the fifth residue of its J segment (39). However, it is clear that all such substitutions need not and probably do not affect antigen binding. For example, the heavy chain from the *P*-Cho-binding myeloma protein M167 (35) differs from that of S107 at 13 positions (8 in hypervariable regions including a size difference) and yet has an association constant for hapten only slightly lower than S107. We have previously shown that, among anti-1,6-galactan-binding myeloma proteins, as many as eight or nine substitutions may occur in hypervariable regions with no significant effect on hapten affinity or specificity (13). Since these systems, as is the case of most hybridoma systems being examined today, are positively selected by antigen, they will in general reveal only substitutions not producing large changes in antigen binding. The negative selection used in this study permits analysis of changes that produce important phenotypic binding variation. Characterization of additional variants in this system may further define the potential of somatic mutations to introduce changes in antigen-binding specificity.

We thank Dr. M. W. Hunkapiller for his invaluable help in sequencer modifications. We also thank Terry Kelly and Mary Heller for their expert technical assistance. This work was supported by grants from the National Institutes of Health (AI 10702 and AI 5231), the National Science Foundation (PCM 77-25635), and the American Cancer Society (NP-317). A.M.G. is a Predoctoral Trainee supported by the National Cancer Institute (5T32-CA09172-06).

1. Hood, L., Loh, E., Hubert, J., Barstad, P., Eaton, B., Early, P. W., Fuhrman, J., Johnson, N., Kronenberg, M. & Schilling, J. (1976) *Cold Spring Harbor Symp. Quant. Biol.* **41**, 817–836.
2. Potter, M. (1977) *Adv. Immunol.* **25**, 141–211.
3. Seidman, J. G., Leder, A., Nau, M., Norman, B. & Leder, P. (1978) *Science* **202**, 11–17.
4. Lenhard-Schuller, R., Hohn, B., Brack, C., Hirama, M. & Tonegawa, S. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4709–4713.
5. Cory, S. & Adams, J. M. (1980) *Cell* **19**, 37–51.
6. Rabbitts, T. H., Matthyssens, G. & Hamlyn, P. H. (1980) *Nature (London)* **284**, 238–243.
7. Davis, M. M., Calame, K., Early, P. W., Livant, D. L., Joho, R., Weissman, I. L. & Hood, L. (1980) *Nature (London)* **283**, 733–739.

8. Max, E., Seidman, J. G. & Leder, P. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3450–3454.
9. Sakano, H., Huppi, K., Heinrich, G. & Tonegawa, S. (1979) *Nature (London)* **280**, 288–294.
10. Early, P. W., Huang, H., Davis, M. M., Calame, K. & Hood, L. (1980) *Cell* **19**, 981–992.
11. Sakano, H., Maki, R., Kurosawa, Y., Roeder, W. & Tonegawa, S. (1980) *Nature (London)* **286**, 676–683.
12. Sakano, H., Kurosawa, Y., Weigert, M. & Tonegawa, S. (1981) *Nature (London)* **290**, 562–565.
13. Rudikoff, S., Rao, D. N., Glaudemans, C. P. J. & Potter, M. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4270–4274.
14. Max, E., Seidman, J. G., Miller, H. & Leder, P. (1980) *Cell* **21**, 793–799.
15. Weigert, M., Perry, R., Kelley, D., Hunapiller, T., Schilling, J. & Hood, L. (1980) *Nature (London)* **283**, 497–499.
16. Gough, N. M. & Bernard, O. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 509–513.
17. Kwan, S. P., Max, E., Seidman, J. G., Leder, P. & Scharff, M. D. *Cell* **26**, 57–66.
18. Weigert, M., Cesari, I. M., Yonkovich, S. J. & Cohn, M. (1970) *Nature (London)* **228**, 1045–1047.
19. Cesari, I. M., & Weigert, M. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 2112–2116.
20. Bernard, O., Hozumi, N. & Tonegawa, S. (1978) *Cell* **15**, 1133–1144.
21. Weigert, M., Gatmaitan, L., Loh, E., Schilling, J. & Hood, L. (1978). *Nature (London)* **291**, 29–34.
22. Gearhart, P., Johnson, N. D., Douglas, R. & Hood, L. (1981) *Nature (London)* **291**, 29–34.
23. Crews, S., Griffin, J., Huang, H., Calame, K. & Hood, L. (1981) *Cell* **25**, 59–66.
24. Bothwell, A., Paskind, M., Reth, M., Imanishi-Kari, T., Rajewsky, K. & Baltimore, D. (1981) *Cell* **24**, 625–637.
25. Kohler, H. (1975) *Transplant. Rev.* **27**, 24–56.
26. Segal, D. M., Padlan, E. A., Cohen, G. H., Rudikoff, S., Potter, M. & Davies, D. R. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 4298–4302.
27. Padlan, E. A., Davies, D. R., Rudikoff, S. & Potter, M. (1976) *Immunochemistry* **13**, 945–949.
28. Cook, W. D. & Scharff, M. D. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5687–5691.
29. Chesebro, B. & Metzger, H. (1972) *Biochemistry II* **11**, 766–771.
30. Evans, J., Steel, M. & Arthur, E. (1974) *Cell* **3**, 153–158.
31. Pierce, S. & Klinman, N. (1976) *J. Exp. Med.* **144**, 1254–1262.
32. Yelton, D. E., Desaymard, C. & Scharff, M. D. (1981) *Hybridoma* **1**, 5–11.
33. Colowick, S. P. & Womack, F. C. (1969) *J. Biol. Chem.* **244**, 774–777.
34. Manjula, B. N., Glaudemans, C. P. J., Mushinski, E. B. & Potter, M. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 932–936.
35. Rudikoff, S. & Potter, M. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2109–2112.
36. Wittmann-Liebold, B., Graffunder, H. & Kohls, H. (1976) *Anal. Biochem.* **75**, 621–633.
37. Hunkapiller, M. W. & Hood, L. (1978) *Biochemistry* **17**, 2124–2133.
38. Rudikoff, S. & Potter, M. (1978) *Biochemistry* **17**, 2703–2707.
39. Cook, W. D., Rudikoff, S., Giusti, A. & Scharff, M. D. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 1240–1244.
40. Kabat, E. A., Wu, T. T. & Bilofsky, H. (1979) *Sequences of Immunoglobulin Chains* (U.S. Dept. of Health, Education, and Welfare), Publication No. 80, p. 2008.
41. Kabat, E. A., Wu, T. T. & Bilofsky, H. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 617–619.
42. Barstad, P., Rudikoff, S., Potter, M., Cohn, M., Konigsberg, W. & Hood, L. (1974) *Science* **183**, 962–964.
43. Rudikoff, S., Satow, Y., Padlan, E., Davies, D. & Potter, M. (1981) *Immunochemistry* **18**, 705–711.
44. Kwan, S. P., Rudikoff, S., Seidman, J. G., Leder, P. & Scharff, M. D. (1981) *J. Exp. Med.* **153**, 1366–1370.
45. Rudikoff, S., Potter, M., Segal, D. M., Padlan, E. A. & Davies, D. R. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 3689–3692.
46. Padlan, E. A., Davies, D. R., Rudikoff, S. & Potter, M. (1976) *Immunochemistry* **13**, 945–949.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/62, C07K 16/28, 16/46

A1

(11) International Publication Number: **WO 98/23761**

(43) International Publication Date: 4 June 1998 (04.06.98)

(21) International Application Number: PCT/US97/19041

(22) International Filing Date: 20 October 1997 (20.10.97)

(30) Priority Data:
08/757,205 27 November 1996 (27.11.96) US

(71) Applicant: GENENTECH, INC. [US/US]; 1 DNA Way, South San Francisco, CA 94080–4990 (US).

(72) Inventors: JARDIEU, Paula, M.; 1896 Pacific Avenue #104, San Francisco, CA 94109 (US). PRESTA, Leonard, G.; 1900 Gough Street #206, San Francisco, CA 94109 (US).

(74) Agents: LEE, Wendy, M. et al.; Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080–4990 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: HUMANIZED ANTI–CD11a ANTIBODIES

(57) Abstract

Humanized anti–CD11a antibodies and various uses therefor are disclosed. The humanized anti–CD11a antibody may bind specifically to human CD11a I–domain, have an IC50(nM) value of no more than about 1nM for preventing adhesion of Jurkat cells to normal human epidermal keratinocytes expressing ICAM–1, and/or an IC50(nM) value of no more than about 1nM in the mixed lymphocyte response assay.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

HUMANIZED ANTI-CD11a ANTIBODIES

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates generally to humanized anti-CD11a antibodies.

Description of Related Art

Lymphocyte function-associated antigen 1 (LFA-1; CD11a/CD18) is involved in leukocyte adhesion during cellular interactions essential for immunologic responses and inflammation (Larson *et al., Immunol. Rev.* 114:181-217 (1990)). LFA-1 is a member of the β2 integrin family and consists of a unique α subunit, CD11a, and a β subunit, CD18, common to other β2 integrin receptors Mac-1 and p150,95. The ligands of LFA-1 include intercellular adhesion molecule-1, ICAM-1, expressed on leukocytes, endothelium, and dermal fibroblasts (Dustin *et al., J. Immunol.* 137:245-254 (1986)), ICAM-2 expressed on resting endothelium and lymphocytes (de Fougerolles *et al., J. Exp. Med.* 174:253-267 (1991)), and ICAM-3 expressed on monocytes and resting lymphocytes (de Fougerolles *et al., J. Exp. Med.* 179:619-629 (1994)).

Monoclonal antibodies (MAbs) against LFA-1 and the ICAMs have been shown, *in vitro*, to inhibit several T cell-dependent immune functions including T cell activation (Kuypers *et al., Res. Immunol.* 140:461(1989)), T cell-dependent B cell proliferation (Fischer *et al., J. Immunol.* 136:3198-3203 (1986)), target cell lysis (Krensky *et al., J. Immunol.* 131:611-616 (1983)), and adhesion of T cells to vascular endothelium (Lo *et al., J. Immunol.* 143:3325-3329 (1989)). In mice, anti-CD11a MAbs induce tolerance to protein antigens (Tanaka *et al., Eur. J. Immunol.* 25:1555-1558 (1995)) and prolong survival of cardiac (Cavazzana-Calvo *et al.,Transplantation* 59:1576-1582 (1995); Nakakura *et al.,Transplantation* 55:412-417 (1993)), bone marrow (Cavazzana-Calvo *et al.,Transplantation* 59:1576-1582 (1995); van Dijken *et al.,Transplantation* 49:882-886 (1990)), corneal (He *et al.,Invest. Opthamol. Vis. Sci.* 35:3218-3225 (1994)), islet (Nishihara *et al., Transplantation Proc.* 27:372 (1995)) and thyroid (Talento *et al., Transplantation* 55:418-422 (1993)) allografts.

In humans, anti-CD11a MAbs prevent graft failure after bone marrow transplantation (Fischer *et al., Blood* 77:249-256 (1991); Stoppa *et al., Transplant Intl.* 4:3-7 (1991)) and preliminary clinical studies of renal allografts treated prophylactically with anti-CD11a MAb, in addition to corticosteroids and azathioprine, are promising (Hourmant *et al., Transplantation* 58:377-380 (1994)). Current therapies against graft rejection include use of OKT3, a murine anti-human CD3 MAb, and cyclosporin A. OKT3 therapy is effective but has several undesirable side effects; its use results in the release of numerous cytokines including tumor necrosis factor-α, interferon-γ, interleukin-2, and interleukin-6, resulting in fever, chills and gastrointestinal distress (for a review see Parlevliet *et al., Transplant Intl.* 5:234-246 (1992); Dantal *et al., Curr. Opin. Immunol.* 3:740-747 (1991)). Cyclosporin A is effective but also has serious side effects (for a review see Barry, *Drugs*, 44:554-566 (1992)).

SUMMARY OF THE INVENTION

The instant invention provides humanized anti-CD11a antibodies. Preferred antibodies bind to the I-domain of human CD11a (*e.g.* to "epitope MHM24" as herein defined) and/or bind CD11a with an affinity of about 1 x 10^{-8}M or stronger. In preferred embodiments, the antibody has an IC50 (nM) value of no more than about 1nM for preventing adhesion of Jurkat cells to normal human epidermal keratinocytes expressing ICAM-1. Preferred humanized antibodies are those which have an IC50 (nM) value of no more than about

1nM in the mixed lymphocyte response (MLR) assay. This IC50 for a humanized antibody in the MLR assay is significantly better than that that for murine MAb 25.3, which has been previously tested *in vivo* (Fischer *et al., Blood* 77:249-256 (1991); Stoppa *et al., Transplant Intl.* 4:3-7 (1991); Hourmant *et al., Transplantation* 58:377-380 (1994)).

The humanized anti-CD11a antibody may have a heavy chain variable region comprising the amino acid sequence of CDR1 (GYSFTGHWMN; SEQ ID NO:10) and/or CDR2 (MIHPSDSETRYNQKFKD; SEQ ID NO:11) and/or CDR3 (GIYFYGTTYFDY; SEQ ID NO:12) of humanized antibody MHM24 F(ab)-8 in Fig. 1 and/or a light chain variable region comprising the amino acid sequence of CDR1 (RASKTISKYLA; SEQ ID NO:13) and/or CDR2 (SGSTLQS; SEQ ID NO:14) and/or CDR3 (QQHNEYPLT; SEQ ID NO:15) of humanized antibody MHM24 F(ab)-8 in Fig. 1. In other embodiments, the antibody comprises an amino acid sequence variant of one or more of the CDRs of humanized MHM24 antibody F(ab)-8, which variant comprises one or more amino acid insertion(s) within or adjacent to a CDR residue and/or deletion(s) within or adjacent to a CDR residue and/or substitution(s) of CDR residue(s) (with substitution(s) being the preferred type of amino acid alteration for generating such variants). Such variants will normally having a binding affinity for human CD11a which is no more than about 1×10^{-8}M.

In preferred embodiments, the humanized antibody includes a light chain variable region comprising the amino acid sequence of SEQ ID NO:2 and/or a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:5 of humanized antibody MHM24 F(ab)-8 in Fig. 1 and/or amino acid sequence variants thereof.

As described herein, it has been possible to reengineer a humanized antibody that bound human CD11a antigen, but not significantly to rhesus CD11a antigen, so as to confer an ability to bind to rhesus CD11a (*i.e.* a "rhesusized" antibody). In this embodiment, the antibody which binds rhesus CD11a may, for example, comprise the the CDR2 amino acid sequence in SEQ ID NO:23. The other CDRs may be the same as those for humanized MHM24 antibody F(ab)-8. Thus, the antibody may comprise the amino acid sequence of the "rhesusized" heavy chain in SEQ ID NO:24, optionally combined with a light chain comprising the amino acid sequence in SEQ ID NO:2.

Various forms of the antibody are contemplated herein. For example, the anti-CD11a antibody may be a full length antibody (*e.g.* having a human immunoglobulin constant region) or an antibody fragment (*e.g.* a $F(ab')_2$). Furthermore, the antibody may be labeled with a detectable label, immobilized on a solid phase and/or conjugated with a heterologous compound (such as a cytotoxic agent).

Diagnostic and therapeutic uses for the antibody are contemplated. In one diagnostic application, the invention provides a method for determining the presence of CD11a protein comprising exposing a sample suspected of containing the CD11a protein to the anti-CD11a antibody and determining binding of the antibody to the sample. For this use, the invention provides a kit comprising the antibody and instructions for using the antibody to detect the CD11a protein.

The invention further provides: isolated nucleic acid encoding the antibody; a vector comprising that nucleic acid, optionally operably linked to control sequences recognized by a host cell transformed with the vector; a host cell comprising that vector; a process for producing the antibody comprising culturing the host cell so that the nucleic acid is expressed and, optionally, recovering the antibody from the host cell culture (*e.g.*

from the host cell culture medium). The invention also provides a composition comprising the humanized anti-CD11a antibody and a pharmaceutically acceptable carrier or diluent. This composition for therapeutic use is sterile and may be lyophilized. The invention further provides a method for treating a mammal suffering from a LFA-1 mediated disorder, comprising administering a pharmaceutically effective amount of the humanized anti-CD11a antibody to the mammal. For such therapeutic uses, other immunosuppressive agents or adhesion molecule antagonists (*e.g.* another LFA-1 antagonist or a VLA-4 antagonist) may be co-administered to the mammal either before, after, or simultaneously with, the humanized anti-CD11a antibody.

Brief Description of the Drawings

Figure 1A shows the amino acid sequences of murine MHM24 light chain (SEQ ID NO: 1), humanized MHM24 F(ab)-8 light chain (SEQ ID NO:2), human consensus sequences of light chain subgroup κI (humκI) (SEQ ID NO:3).

Figure 1B shows the amino acid sequences of murine MHM24 heavy chain (SEQ ID NO:4), humanized MHM24 F(ab)-8 heavy chain(SEQ ID NO:5), human consensus sequences of heavy chain subgroup III (humIII) (SEQ ID NO:6) and "rhesusized" antibody mutant heavy chain of the Example (SEQ ID NO:24).

In Figs. 1A and 1B, hypervariable regions based on sequence hypervariability (Kabat *et al., Sequences of Proteins of Immunological Interest.* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) are enclosed within brackets and hypervariable loops based on structure of F(ab)-antigen complexes (Chothia *et al., Nature* 342:8767 (1989)) are in italics. Residue numbering is according to Kabat *et al.,* with insertions shown as a, b, and c.

Figure 2 shows sequences of human CD11a I-domain (SEQ ID NO:7) and rhesus CD11a I-domain (SEQ ID NO:8). β-strands and α-helices are underlined and labeled according to Qu *et al., Proc. Natl. Acad. Sci.* 92:10277-10281 (1995). The rhesus I-domain sequence (rhCD11a) shows only the four differences from human I-domain. The binding epitope for the MHM24 MAb (SEQ ID NO:9) is shown in bold (Champe *et al., J. Biol. Chem.* 270:1388-1394 (1995)).

Figure 3 depicts inhibition of human Jurkat T-cells to normal human keratinocytes by murine MHM24 (filled circles), chimeric MHM24 (open triangles), humanized MHM24 (HuIgG1) (filled squares), and a human IgG1 isotype control (+). Percent binding measured by fluorescence of labeled Jurkat cells.

Figures 4A-4C show inhibition of binding of rhesus lymphocytes to normal human keratinocytes (Fig. 4A), rhesus lymphocytes to recombinant human ICAM-1 coated on plates (Fig. 4B), and rhesus/human CD11a chimera-transfected 293 cells to normal human keratinocytes (Fig. 4C). Inhibition by rhesus-binding MHM24 (RhIgG1) (filled squares), anti-CD18 MHM23 (filled circles), a human IgG1 isotype control (+) (Figs. 4A and 4C), and a murine IgG1 isotype control (+) (Fig. 4B). Percent binding measured by fluorescence of labeled lymphocytes (Figs. 4A and B) or labeled 293 cells (Fig. 4C).

Figure 5 shows human mixed lymphocyte response assy (MLR) is blocked by murine MHM24 (filled circles), humanized MHM24 (HuIgG1) (filled squares), and a humanized isotype IgG1 control (filled diamond). Percent stimulation index (%SI) is the ratio of the response at a given MAb concentration to the maximal response with no MAb present. Data is representative of multiple assays using at least two different stimulator/responder pairs.

Detailed Description of the Preferred Embodiments

I. Definitions

Unless indicated otherwise, the term "CD11a" when used herein refers to the alpha subunit of LFA-1 from any mammal, but preferably from a human. The CD11a may be isolated from a natural source of the molecule or may be produced by synthetic means (*e.g.*, using recombinant DNA technology.) The amino acid sequence for human CD11a is described in EP 362 526B1, for example.

The term "I-domain" of CD11a refers to the region of this molecule delineated in Champe *et al., J. Biol. Chem.* 270:1388-1394 (1995) and/or Qu *et al. Proc. Natl. Acad. Sci.* 92:10277-10281 (1995). The amino acid sequences of human CD11a I-domain (SEQ ID NO:7) and rhesus CD11a I-domain (SEQ ID NO:8) are depicted in Fig. 2 herein.

The term "epitope MHM24" when used herein, unless indicated otherwise, refers to the region in the I-domain of human CD11a to which the MHM24 antibody (see Example below) binds. This epitope comprises the amino acid sequence of SEQ ID NO:9 and, optionally, other amino acid residues of CD11a and/or CD18.

The term "LFA-1-mediated disorder" refers to a pathological state caused by cell adherence interactions involving the LFA-1 receptor on lymphocytes. Examples of such disorders include T cell inflammatory responses such as inflammatory skin diseases including psoriasis; responses associated with inflammatory bowel disease (such as Crohn's disease and ulcerative colitis); adult respiratory distress syndrome; dermatitis; meningitis; encephalitis; uveitis; allergic conditions such as eczema and asthma; conditions involving infiltration of T cells and chronic inflammatory responses; skin hypersensitivity reactions (including poison ivy and poison oak); atherosclerosis; leukocyte adhesion deficiency; autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus (SLE), diabetes mellitus, multiple sclerosis, Reynaud's syndrome, autoimmune thyroiditis, experimental autoimmune encephalomyelitis, Sjorgen's syndrome, juvenile onset diabetes, and immune responses associated with delayed hypersensitivity mediated by cytokines and T-lymphocytes typically found in tuberculosis, sarcoidosis, polymyositis, granulomatosis and vasculitis; pernicious anemia; chronic obstructive pulmonary disease (COPD); bronchitis; insulinitis; rhinitis; urticaria; glomerulonephritis; diseases involving leukocyte diapedesis; CNS inflammatory disorder; multiple organ injury syndrome secondary to septicaemia or trauma; autoimmune hemolytic anemia; myethemia gravis; antigen-antibody complex mediated diseases; nephrotic syndrome; malignancies (*e.g.*, B-cell malignancies such as chronic lymphocytic leukemia or hairy cell leukemia); all types of transplantations, including graft vs. host or host vs. graft disease; HIV and rhinovirus infection; pulmonary fibrosis; invasion of tumor cells into secondary organs etc.

The term "immunosuppressive agent" as used herein for adjunct therapy refers to substances that act to suppress or mask the immune system of the host into which the graft is being transplanted. This would include substances that suppress cytokine production, downregulate or suppress self-antigen expression, or mask the MHC antigens. Examples of such agents include 2-amino-6-aryl-5-substituted pyrimidines (see U.S. Pat. No. 4,665,077), azathioprine (or cyclophosphamide, if there is an adverse reaction to azathioprine); bromocryptine; glutaraldehyde (which masks the MHC antigens, as described in U.S. Pat. No. 4,120,649); anti-idiotypic antibodies for MHC antigens and MHC fragments; cyclosporin A; steroids such as glucocorticosteroids, *e.g.*, prednisone, methylprednisolone, and dexamethasone; cytokine or cytokine receptor

antagonists including anti-interferon-γ, -β, or -α antibodies; anti-tumor necrosis factor-α antibodies; anti-tumor necrosis factor-β antibodies; anti-interleukin-2 antibodies and anti-IL-2 receptor antibodies; anti-L3T4 antibodies; heterologous anti-lymphocyte globulin; pan-T antibodies, preferably anti-CD3 or anti-CD4/CD4a antibodies; soluble peptide containing a LFA-3 binding domain (WO 90/08187 published 7/26/90); streptokinase; TGF-β; streptodornase; RNA or DNA from the host; FK506; RS-61443; deoxyspergualin; rapamycin; T-cell receptor (U.S. Pat. No. 5,114,721); T-cell receptor fragments (Offner *et al., Science* 251:430-432 (1991); WO 90/11294; and WO 91/01133); and T cell receptor antibodies (EP 340,109) such as T10B9. These agents are administered at the same time or at separate times from the CD11a antibody, and are used at the same or lesser dosages than as set forth in the art. The preferred adjunct immunosuppressive agent will depend on many factors, including the type of disorder being treated including the type of transplantation being performed, as well as the patient's history, but a general overall preference is that the agent be selected from cyclosporin A, a glucocorticosteroid (most preferably prednisone or methylprednisolone), OKT-3 monoclonal antibody, azathioprine, bromocryptine, heterologous anti-lymphocyte globulin, or a mixture thereof.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, *etc.* Preferably, the mammal is human.

The term "graft" as used herein refers to biological material derived from a donor for transplantation into a recipient. Grafts include such diverse material as, for example, isolated cells such as islet cells and neural-derived cells (*e.g.* schwann cells), tissue such as the amniotic membrane of a newborn, bone marrow, hematopoietic precursor cells, and organs such as skin, heart, liver, spleen, pancreas, thyroid lobe, lung, kidney, tubular organs (*e.g.*, intestine, blood vessels, or esophagus), etc. The tubular organs can be used to replace damaged portions of esophagus, blood vessels, or bile duct. The skin grafts can be used not only for burns, but also as a dressing to damaged intestine or to close certain defects such as diaphragmatic hernia. The graft is derived from any mammalian source, including human, whether from cadavers or living donors. Preferably the graft is bone marrow or an organ such as heart and the donor of the graft and the host are matched for HLA class II antigens.

The term "donor" as used herein refers to the mammalian species, dead or alive, from which the graft is derived. Preferably, the donor is human. Human donors are preferably volunteer blood-related donors that are normal on physical examination and of the same major ABO blood group, because crossing major blood group barriers possibly prejudices survival of the allograft. It is, however, possible to transplant, for example, a kidney of a type O donor into an A, B or AB recipient.

The term "transplant" and variations thereof refers to the insertion of a graft into a host, whether the transplantation is syngeneic (where the donor and recipient are genetically identical), allogeneic (where the donor and recipient are of different genetic origins but of the same species), or xenogeneic (where the donor and recipient are from different species). Thus, in a typical scenario, the host is human and the graft is an

isograft, derived from a human of the same or different genetic origins. In another scenario, the graft is derived from a species different from that into which it is transplanted, such as a baboon heart transplanted into a human recipient host, and including animals from phylogenically widely separated species, for example, a pig heart valve, or animal beta islet cells or neuronal cells transplanted into a human host.

"Increasing tolerance of a transplanted graft" by a host refers to prolonging the survival of a graft in a host in which it is transplanted, *i.e.*, suppressing the immune system of the host so that it will better tolerate a foreign transplant.

"Intermittent" or "periodic" dosing is a dosing that is continuous for a certain period of time and is at regular intervals that are preferably separated by more than one day.

"Selective tolerance" of the disorder refers to a tolerance by the host's immune system for the specific agent causing the disorder, but retaining the ability of the host to reject a second allogeneic or xenogeneic graft. Preferably, the tolerance is such that the immune system is left otherwise intact.

The term "LFA-1 antagonist" refers to a molecule that acts as a competitive inhibitor of the LFA-1 interaction with ICAM-1. Examples of such molecules include antibodies directed against either CD11a (*e.g.*, the humanized anti-CD11a antibodies described herein) or CD18 or both, antibodies to ICAM-1, and other molecules such as peptides (*e.g.*, peptidomimetic antagonists).

The term "VLA-4 antagonist" refers to a molecule that acts as a competitive inhibitor of the VLA-4 interaction with VCAM. Examples of such molecules include antibodies directed against either VLA-4 or VCAM and other molecules (*e.g.*, peptidomimetic antagonists).

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al.*, *Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al.*, *Nature* 352:624-628 (1991) and Marks *et al.*, *J. Mol. Biol.* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; and Morrison *et al., Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (*i.e.* residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat *et al., Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) and/or those residues from a "hypervariable loop" (*i.e.* residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

"Humanized" forms of non-human (*e.g.,* murine) antibodies are chimeric antibodies which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which hypervariable region residues of the recipient are replaced by hypervariable region residues from a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al., Nature* 321:522-525 (1986); Reichmann *et al., Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H - V_L). By using a linker that is too short to allow pairing between the two

domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al., Proc. Natl. Acad. Sci. USA* 90:6444-6448 (1993).

The expression "linear antibodies" when used throughout this application refers to the antibodies described in Zapata *et al. Protein Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody *in situ* within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The term "epitope tagged" when used herein refers to the anti-CD11a antibody fused to an "epitope tag". The epitope tag polypeptide has enough residues to provide an epitope against which an antibody thereagainst can be made, yet is short enough such that it does not interfere with activity of the CD11a antibody. The epitope tag preferably is sufficiently unique so that the antibody thereagainst does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least 6 amino acid residues and usually between about 8-50 amino acid residues (preferably between about 9-30 residues). Examples include the flu HA tag polypeptide and its antibody 12CA5 (Field *et al. Mol. Cell. Biol.* 8:2159-2165 (1988)); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan *et al., Mol. Cell. Biol.* 5(12):3610-3616 (1985)); and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody (Paborsky *et al., Protein Engineering* 3(6):547-553 (1990)). In certain embodiments, the epitope tag is a "salvage receptor binding epitope".

As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (*e.g.*, IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (*e.g.*, I^{131}, I^{125}, Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include Adriamycin, Doxorubicin, 5-Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiotepa, Taxotere (docetaxel), Busulfan, Cytoxin, Taxol, Methotrexate, Cisplatin, Melphalan, Vinblastine, Bleomycin, Etoposide, Ifosfamide, Mitomycin C, Mitoxantrone, Vincreistine,

Vinorelbine, Carboplatin, Teniposide, Daunomycin, Carminomycin, Aminopterin, Dactinomycin, Mitomycins, Esperamicins (see U.S. Pat. No. 4,675,187), Melphalan and other related nitrogen mustards.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, *e.g.*, Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella *et al.*, "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt *et al.*, (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the antibody. The label may itself be detectable by itself (*e.g.*, radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

By "solid phase" is meant a non-aqueous matrix to which the antibody of the present invention can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (*e.g.* controlled pore glass), polysaccharides (*e.g.*, agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (*e.g.* an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Patent No. 4,275,149.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-CD11a antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

An "isolated" nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the antibody nucleic acid. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the antibody where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The expression "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for

prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

II. Modes for Carrying out the Invention

A. Antibody Preparation

A method for humanizing a nonhuman CD11a antibody is described in the Example below. In order to humanize an anti-CD11a antibody, the nonhuman antibody starting material is prepared. Exemplary techniques for generating such antibodies will be described in the following sections.

(i) Antigen preparation.

The CD11a antigen to be used for production of antibodies may be, *e.g.*, a soluble form of the extracellular domain of CD11a or other fragment of CD11a (*e.g.* a CD11a fragment comprising the "MHM24 epitope", such as CD11a I-domain fragment). Alternatively, cells expressing CD11a at their cell surface can be used to generate antibodies. Such cells can be transformed to express CD11a and, optionally, CD18 or may be other naturally occurring cells (*e.g.* human lymphoblastoid cells, see Hildreth *et al. Eur. J. Immunol.* 13:202-208 (1983)) or Jurkat cells (see Example below). Other forms of CD11a useful for generating antibodies will be apparent to those skilled in the art.

(ii) Polyclonal antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, *e.g.*, 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(iii) Monoclonal antibodies

Monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al.*, *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOP-21 and M.C.-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson *et al.*, *Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Recombinant production of antibodies will be described in more detail below.

(iv) Humanization and amino acid sequence variants

The Example below describes a procedure for humanization of an anti-CD11a antibody. In certain embodiments, it may be desirable to generate amino acid sequence variants of the humanized antibody, particularly where these improve the binding affinity or other biological properties of the humanized antibody.

Amino acid sequence variants of humanized anti-CD11a antibody are prepared by introducing appropriate nucleotide changes into the humanized anti-CD11a antibody DNA, or by peptide synthesis. Such variants include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences shown for the humanized anti-CD11a F(ab)-8 (*e.g.* as in SEQ ID NO's 2 & 5). Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the humanized anti-CD11a antibody, such as changing the number or position of glycosylation sites.

A useful method for identification of certain residues or regions of the humanized anti-CD11a antibody polypeptide that are preferred locations for mutagenesis is called "alanine scanning mutagenesis," as described by Cunningham and Wells *Science*, 244:1081-1085 (1989). Here, a residue or group of target residues are identified (*e.g.*, charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with CD11a antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed humanized anti-CD11a antibody variants are screened for the desired activity.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include humanized anti-CD11a antibody with an N-terminal methionyl residue or the antibody fused to an epitope tag. Other insertional variants of the humanized anti-CD11a antibody molecule include the fusion to the N- or C-terminus of humanized anti-CD11a antibody of an enzyme or a polypeptide which increases the serum half-life of the antibody (see below).

Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the humanized anti-CD11a antibody molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include the hypervariable loops, but FR alterations are also contemplated. Table IV in the Example below provides guidance as to hypervariable region residues which can be altered. Hypervariable region residues or FR residues involved in antigen binding are generally substituted in a relatively conservative manner. Such conservative substitutions are shown in Table I under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table I, or as further described below in reference to amino acid classes, are introduced and the products screened.

Table I

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; lys; arg	gln
Asp (D)	glu	glu
Cys (C)	ser	ser
Gln (Q)	asn	asn
Glu (E)	asp	asp
Gly (G)	pro; ala	ala
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	leu
Pro (P)	ala	ala
Ser (S)	thr	thr

Thr (T)	ser	ser
Trp (W)	tyr; phe	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophilic: cys, ser, thr;

(3) acidic: asp, glu;

(4) basic: asn, gln, his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class. Any cysteine residue not involved in maintaining the proper conformation of the humanized anti-CD11a antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

Another type of amino acid variant of the antibody alters the original glycosylation pattern of the antibody. By altering is meant deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody.

Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

Nucleic acid molecules encoding amino acid sequence variants of humanized anti-CD11a antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by

oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of humanized anti-CD11a antibody.

Ordinarily, amino acid sequence variants of the humanized anti-CD11a antibody will have an amino acid sequence having at least 75% amino acid sequence identity with the original humanized antibody amino acid sequences of either the heavy or the light chain (*e.g.* as in SEQ ID NO:2 or 5), more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, and most preferably at least 95%. Identity or homology with respect to this sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the humanized anti-CD11a residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions (as defined in Table I above) as part of the sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the antibody sequence shall be construed as affecting sequence identity or homology.

(v) Screening for biological properties

Antibodies having the characteristics identified herein as being desirable in a humanized anti-CD11a antibody are screened for.

To screen for antibodies which bind to the epitope on CD11a bound by an antibody of interest (*e.g.*, those which block binding of the MHM24 antibody to CD11a), a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping, *e.g.* as described in Champe *et al., J. Biol. Chem.* 270:1388-1394 (1995), can be performed to determine whether the antibody binds an epitope of interest.

Antibody affinities (*e.g.* for human CD11a or rhesus CD11a) may be determined by saturation binding using either peripheral blood mononuclear cells or rhesus leukocytes as described in the Example below. According to this method for determining antibody affinity, lymphocytes or rhesus leukocytes are added to the plates in a volume of 170 µl per well and plates are incubated for 2 hr at room. After incubation, cells are harvested and washed 10 times. Samples are then counted. Data is transformed from counts per minute to nanomolarity and four-parameter curve-fitting of saturation plots (bound versus total) are then performed to determine K_d (app) values. Preferred humanized antibodies are those which bind human CD11a with a K_d value of no more than about 1×10^{-7}; preferably no more than about 1×10^{-8}; more preferably no more than about 1×10^{-9}; and most preferably no more than about 2×10^{-10}.

It is also desirable to select humanized antibodies which have beneficial anti-adhesion properties in the "keratinocyte monolayer adhesion assay". Preferred antibodies are those which have an IC50 (nM) value of no more than about 250nM; preferably no more than about 100nM; more preferably no more than about 1nM and most preferably no more than about 0.5nM for preventing adhesion of Jurkat cells to normal human epidermal keratinocytes expressing ICAM-1. According to this assay, normal human epidermal keratinocytes are removed from culture flasks and resuspended in lymphocyte assay medium at a concentration of 5×10^5 viable cells/ml. Aliquots of 0.1 ml/well are then cultured overnight in flat-bottom 96-well plates; appropriate wells are stimulated by addition of interferon-gamma at 100 units/well. Jurkat clone E6-1 cells are labeled, washed, resuspended to 1×10^6 cells/ml, and incubated with 2-fold serial dilutions starting at 500ng/ml antibody at 4° C for 30 min. After removal of medium from the keratinocyte monolayer, 0.1 ml/well of labeled

cells are added and incubated at 37°C for 1 h. The wells are washed to remove non-attached cells and fluorescence is measured.

Desirable humanized anti-CDIIa antibodies are those which have an IC50 (nM) value of no more than about 100nM; preferably no more than about 50nM; more preferably no more than about 5nM and most preferably no more than about 1nM in the mixed lymphocyte response (MLR) assay, using human lymphocytes. For both human and rhesus MLR, peripheral blood lymphocytes from two unrelated donors are isolated from whole, heparinized blood and are resuspended to a concentration of 3×10^6 cells/ml in RPMI 1640 (GIBCO) with additives as described in the Example below. The stimulator cells are made unresponsive by irradiation. Responder cells at a concentration of 1.5×10^5 cells per well are co-cultured with an equal number of stimulator cells in 96-well, flat-bottom plates. Two-fold serial dilutions of antibody starting at a concentration of 10nM are added to the cultures to give a total volume of 200 µl/well. The cultures are incubated at 37°C in 5% CO_2 for 5 days and then pulsed with 1 µCi/well of [^{3}H]thymidine for 16 h and [^{3}H]thymidine incorporation is measured.

(vi) Antibody fragments

In certain embodiments, the humanized CD11a antibody is an antibody fragment. Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, *e.g.*, Morimoto *et al.*, *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan *et al.*, *Science* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter *et al.*, *Bio/Technology* 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

(vii) Multispecific antibodies

In some embodiments, it may be desirable to generate multispecific (*e.g.* bispecific) humanized CD11a antibodies having binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the CD11a protein. Alternatively, an anti-CD11a arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (*e.g.*, CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the CD11a-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express CD11a. These antibodies possess an CD11a-binding arm and an arm which binds the cytotoxic agent (*e.g.*, saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (*e.g.*, $F(ab')_2$ bispecific antibodies).

According to another approach for making bispecific antibodies, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (*e.g.*, tyrosine or tryptophan). Compensatory "cavities"

of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (*e.g.*, alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers. See WO96/27011 published September 6, 1996.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan *et al.*, *Science* 229:81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli,* which can be chemically coupled to form bispecific antibodies. Shalaby *et al., J. Exp. Med.* 175:217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling *in vitro* to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the HER2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al., J. Immunol.* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger *et al., Proc. Natl. Acad. Sci. USA* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al., J. Immunol.* 152:5368 (1994). Alternatively, the bispecific antibody may be a "linear antibody" produced as described in Zapata *et al. Protein Eng.* 8(10):1057-1062 (1995).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al., J. Immunol.* 147:60 (1991).

(viii) Other modifications

Other modifications of the humanized anti-CD11a antibody are contemplated. For example, it may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron *et al., J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff *et al., Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson *et al., Anti-Cancer Drug Design* 3:219-230 (1989).

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (*e.g.*, an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (*i.e.*, a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-CD11a antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y and ^{186}Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al., Science* 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

In another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (*e.g.*, avidin) which is conjugated to a cytotoxic agent (*e.g.*, a radio nuclide).

The anti-CD11a antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein *et al., Proc. Natl. Acad. Sci. USA* 82:3688 (1985); Hwang *et al., Proc. Natl Acad. Sci. USA* 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin *et al., J. Biol. Chem.* 257:286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent (such as Doxorubicin) is optionally contained within the liposome. See Gabizon *et al., J. National Cancer Inst.*81(19):1484 (1989)

The antibody of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (*e.g.*, a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Patent No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, *e.g.*, Massey, *Nature* 328:457-458 (1987)). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-CD11a antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, *e.g.*, Neuberger *et al., Nature* 312:604-608 (1984)).

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation

of a salvage receptor binding epitope into the antibody fragment (*e.g.*, by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, *e.g.*, by DNA or peptide synthesis). See WO96/32478 published October 17, 1996.

The salvage receptor binding epitope generally constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (*e.g.*, of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence (5' to 3'): PKNSSMISNTP (SEQ ID NO:16), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:17), HQNLSDGK (SEQ ID NO:18), HQNISDGK (SEQ ID NO:19), or VISSHLGQ (SEQ ID NO:20), particularly where the antibody fragment is a Fab or $F(ab')_2$. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s)(5' to 3'): HQNLSDGK (SEQ ID NO:18), HQNISDGK (SEQ ID NO:19), or VISSHLGQ (SEQ ID NO:20) and the sequence: PKNSSMISNTP (SEQ ID NO:16).

Covalent modifications of the humanized CD11a antibody are also included within the scope of this invention. They may be made by chemical synthesis or by enzymatic or chemical cleavage of the antibody, if applicable. Other types of covalent modifications of the antibody are introduced into the molecule by reacting targeted amino acid residues of the antibody with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues.

Cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysinyl and amino-terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione, and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine

functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R-N=C=N-R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. These residues are deamidated under neutral or basic conditions. The deamidated form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification involves chemically or enzymatically coupling glycosides to the antibody. These procedures are advantageous in that they do not require production of the antibody in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, *CRC Crit. Rev. Biochem.*, pp. 259-306 (1981).

Removal of any carbohydrate moieties present on the antibody may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the antibody to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the antibody intact. Chemical deglycosylation is described by Hakimuddin, *et al. Arch. Biochem. Biophys.* 259:52 (1987) and by Edge *et al. Anal. Biochem.*, 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on antibodies can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al. Meth. Enzymol.* 138:350 (1987).

Another type of covalent modification of the antibody comprises linking the antibody to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

B. Vectors, Host Cells and Recombinant Methods

The invention also provides isolated nucleic acid encoding the humanized anti-CD11a antibody, vectors and host cells comprising the nucleic acid, and recombinant techniques for the production of the antibody.

For recombinant production of the antibody, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the monoclonal antibody is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(i) Signal sequence component

The anti-CD11a antibody of this invention may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which is preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. The heterologous signal sequence selected preferably is one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native anti-CD11a antibody signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, *e.g.*, the yeast invertase leader, α factor leader (including *Saccharomyces* and *Kluyveromyces* α-factor leaders), or acid phosphatase leader, the *C. albicans* glucoamylase leader, or the signal described in WO 90/13646. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available.

The DNA for such precursor region is ligated in reading frame to DNA encoding the anti-CD11a antibody.

(ii) Origin of replication component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

(iii) Selection gene component

Expression and cloning vectors may contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin,

neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin, mycophenolic acid and hygromycin.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the anti-CD11a antibody nucleic acid, such as DHFR, thymidine kinase, metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, *etc.*

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity.

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding anti-CD11a antibody, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, *e.g.*, kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid YRp7 (Stinchcomb *et al., Nature*, 282:39 (1979)). The *trp*1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1. Jones, *Genetics*, 85:12 (1977). The presence of the *trp*1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu*2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu*2 gene.

In addition, vectors derived from the 1.6 μm circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts. Alternatively, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis*. Van den Berg, *Bio/Technology*, 8:135 (1990). Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed. Fleer *et al., Bio/Technology*, 9:968-975 (1991).

(iv) Promoter component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the anti-CD11a antibody nucleic acid. Promoters suitable for use with prokaryotic hosts include the *phoA* promoter , β-lactamase and lactose promoter systems, alkaline phosphatase, a tryptophan (trp) promoter system, and hybrid promoters such as the tac promoter. However, other known bacterial promoters are suitable. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the anti-CD11a antibody.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence

found 70 to 80 bases upstream from the start of transcription of many genes is a CNCAAT region where N may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase or other glycolytic enzymes, such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

Anti-CD11a antibody transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, *e.g.*, the actin promoter or an immunoglobulin promoter, from heat-shock promoters, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978. See also Reyes *et al.*, *Nature* 297:598-601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus. Alternatively, the rous sarcoma virus long terminal repeat can be used as the promoter.

(v) Enhancer element component

Transcription of a DNA encoding the anti-CD11a antibody of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature* 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the anti-CD11a antibody-encoding sequence, but is preferably located at a site 5' from the promoter.

(vi) Transcription termination component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding anti-CD11a antibody. One useful transcription termination component is the bovine growth hormone polyadenylation region. See WO94/11026 and the expression vector disclosed therein.

(vii) Selection and transformation of host cells

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia, e.g., E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, e.g., Salmonella typhimurium, Serratia, e.g., Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (*e.g., B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for anti-CD11a antibody-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces* hosts such as, *e.g., K. lactis, K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa*; *Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, *e.g., Neurospora, Penicillium, Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger*.

Suitable host cells for the expression of glycosylated anti-CD11a antibody are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, *e.g.*, the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey

kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al., J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub *et al., Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al., Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for anti-CD11a antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

(viii) Culturing the host cells

The host cells used to produce the anti-CD11a antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham *et al., Meth. Enz.* 58:44 (1979), Barnes *et al., Anal. Biochem.*102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

(ix) Purification of anti-CD11a antibody

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter *et al., Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli.* Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark *et al., J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human γ3 (Guss *et al., EMBO J.* 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™resin (J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (*e.g.*, from about 0-0.25M salt).

C. Pharmaceutical Formulations

Therapeutic formulations of the antibody are prepared for storage by mixing the antibody having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.*, Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide an immunosuppressive agent.[1] Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and

poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, *e.g.*, films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the Lupron Depot™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

D. Non-therapeutic Uses for the Antibody

The antibodies of the invention may be used as affinity purification agents. In this process, the antibodies are immobilized on a solid phase such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody is contacted with a sample containing the CD11a protein (or fragment thereof) to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the CD11a protein, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent, such as glycine buffer, pH 5.0, that will release the CD11a protein from the antibody.

Anti-CD11a antibodies may also be useful in diagnostic assays for CD11a protein, *e.g.*, detecting its expression in specific cells, tissues, or serum.

For diagnostic applications, the antibody typically will be labeled with a detectable moiety. Numerous labels are available which can be generally grouped into the following categories:

(a) Radioisotopes, such as ^{35}S, ^{14}C, ^{125}I, ^{3}H, and ^{131}I. The antibody can be labeled with the radioisotope using the techniques described in *Current Protocols in Immunology*, Volumes 1 and 2, Coligen *et al.*, Ed. Wiley-Interscience, New York, New York, Pubs. (1991) for example and radioactivity can be measured using scintillation counting.

(b) Fluorescent labels such as rare earth chelates (europium chelates) or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, Lissamine, phycoerythrin and Texas Red are available. The fluorescent

labels can be conjugated to the antibody using the techniques disclosed in *Current Protocols in Immunology, supra*, for example. Fluorescence can be quantified using a fluorimeter.

(c) Various enzyme-substrate labels are available and U.S. Patent No. 4,275,149 provides a review of some of these. The enzyme generally catalyzes a chemical alteration of the chromogenic substrate which can be measured using various techniques. For example, the enzyme may catalyze a color change in a substrate, which can be measured spectrophotometrically. Alternatively, the enzyme may alter the fluorescence or chemiluminescence of the substrate. Techniques for quantifying a change in fluorescence are described above. The chemiluminescent substrate becomes electronically excited by a chemical reaction and may then emit light which can be measured (using a chemiluminometer, for example) or donates energy to a fluorescent acceptor. Examples of enzymatic labels include luciferases (*e.g.*, firefly luciferase and bacterial luciferase; U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, malate dehydrogenase, urease, peroxidase such as horseradish peroxidase (HRPO), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases (*e.g.*, glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase), heterocyclic oxidases (such as uricase and xanthine oxidase), lactoperoxidase, microperoxidase, and the like. Techniques for conjugating enzymes to antibodies are described in O'Sullivan *et al.*, Methods for the Preparation of Enzyme-Antibody Conjugates for use in Enzyme Immunoassay, in *Methods in Enzym.* (ed J. Langone & H. Van Vunakis), Academic press, New York, 73:147-166 (1981).

Examples of enzyme-substrate combinations include, for example:

(i) Horseradish peroxidase (HRPO) with hydrogen peroxidase as a substrate, wherein the hydrogen peroxidase oxidizes a dye precursor (*e.g.*, orthophenylene diamine (OPD) or 3,3',5,5'-tetramethyl benzidine hydrochloride (TMB));

(ii) alkaline phosphatase (AP) with para-Nitrophenyl phosphate as chromogenic substrate; and

(iii) β-D-galactosidase (β-D-Gal) with a chromogenic substrate (*e.g.*, p-nitrophenyl-β-D-galactosidase) or fluorogenic substrate 4-methylumbelliferyl-β-D-galactosidase.

Numerous other enzyme-substrate combinations are available to those skilled in the art. For a general review of these, see U.S. Patent Nos. 4,275,149 and 4,318,980.

Sometimes, the label is indirectly conjugated with the antibody. The skilled artisan will be aware of various techniques for achieving this. For example, the antibody can be conjugated with biotin and any of the three broad categories of labels mentioned above can be conjugated with avidin, or *vice versa*. Biotin binds selectively to avidin and thus, the label can be conjugated with the antibody in this indirect manner. Alternatively, to achieve indirect conjugation of the label with the antibody, the antibody is conjugated with a small hapten (*e.g.*, digoxin) and one of the different types of labels mentioned above is conjugated with an anti-hapten antibody (*e.g.*, anti-digoxin antibody). Thus, indirect conjugation of the label with the antibody can be achieved.

In another embodiment of the invention, the anti-CD11a antibody need not be labeled, and the presence thereof can be detected using a labeled antibody which binds to the CD11a antibody.

The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, *Monoclonal Antibodies: A Manual of Techniques*, pp.147-158 (CRC Press, Inc. 1987).

Competitive binding assays rely on the ability of a labeled standard to compete with the test sample analyte for binding with a limited amount of antibody. The amount of CD11a protein in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies generally are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte which remain unbound.

Sandwich assays involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected. In a sandwich assay, the test sample analyte is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three-part complex. See, *e.g.*, US Pat No. 4,376,110. The second antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

For immunohistochemistry, the tumor sample may be fresh or frozen or may be embedded in paraffin and fixed with a preservative such as formalin, for example.

The antibodies may also be used for *in vivo* diagnostic assays. Generally, the antibody is labeled with a radio nuclide (such as ^{111}In, ^{99}Tc, ^{14}C, ^{131}I, ^{125}I, ^{3}H, ^{32}P or ^{35}S) so that the tumor can be localized using immunoscintiography.

E. Diagnostic Kits

As a matter of convenience, the antibody of the present invention can be provided in a kit, *i.e.*, a packaged combination of reagents in predetermined amounts with instructions for performing the diagnostic assay. Where the antibody is labeled with an enzyme, the kit will include substrates and cofactors required by the enzyme (*e.g.*, a substrate precursor which provides the detectable chromophore or fluorophore). In addition, other additives may be included such as stabilizers, buffers (*e.g.*, a block buffer or lysis buffer) and the like. The relative amounts of the various reagents may be varied widely to provide for concentrations in solution of the reagents which substantially optimize the sensitivity of the assay. Particularly, the reagents may be provided as dry powders, usually lyophilized, including excipients which on dissolution will provide a reagent solution having the appropriate concentration.

F. Therapeutic Uses for the Antibody

It is contemplated that the anti-CD11a antibody of the present invention may be used to treat the various LFA-1 mediated disorders as described herein.

The anti-CD11a antibody is administered by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal, and, if desired for local immunosuppressive treatment, intralesional administration (including perfusing or otherwise contacting the graft with the antibody before transplantation). Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. In addition, the anti-CD11a antibody is suitably administered by pulse infusion, particularly with declining doses of the antibody. Preferably the dosing is given by injections, most preferably intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic.

For the prevention or treatment of disease, the appropriate dosage of antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (*e.g.*, 0.1-20mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays. An exemplary dosing regimen is disclosed in WO 94/04188.

The antibody composition will be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The "therapeutically effective amount" of the antibody to be administered will be governed by such considerations, and is the minimum amount necessary to prevent, ameliorate, or treat the LFA-1-mediated disorder, including treating rheumatoid arthritis, reducing inflammatory responses, inducing tolerance of immunostimulants, preventing an immune response that would result in rejection of a graft by a host or vice-versa, or prolonging survival of a transplanted graft. Such amount is preferably below the amount that is toxic to the host or renders the host significantly more susceptible to infections.

The antibody need not be, but is optionally formulated with one or more agents currently used to prevent or treat the disorder in question. For example, in rheumatoid arthritis, the antibody may be given in conjunction with a glucocorticosteroid. In addition, T cell receptor peptide therapy is suitably an adjunct therapy to prevent clinical signs of autoimmune encephalomyelitis. For transplants, the antibody may be administered concurrently with or separate from an immunosuppressive agent as defined above, *e.g.*, cyclosporin A, to modulate the immunosuppressant effect. Alternatively, or in addition, VLA-4 antagonists or other LFA-1 antagonists may be administered to the mammal suffering from a LFA-1 mediated disorder. The effective amount of such other agents depends on the amount of anti-CD11a antibody present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as used hereinbefore or about from 1 to 99% of the heretofore employed dosages.

G. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container and a label. Suitable containers include, for example, bottles, vials, syringes, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is

effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The active agent in the composition is the anti-CD11a antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

EXAMPLE

PRODUCTION OF HUMANIZED ANTI-CD11a ANTIBODIES

This example describes the humanization and *in vitro* biological efficacy of a murine anti-human CD11a monoclonal antibody, MHM24 (Hildreth *et al., Eur. J. Inmunol.* 13:202-208 (1983)). Previous studies on the murine MHM24 have shown that it, like other anti-CD11a antibodies, can inhibit T cell function (Hildreth *et al., J. Immunol.* 134:3272-3280 (1985); Dougherty *et al., Eur. J. Immunol.* 17:943-947 (1987)). Both the murine and humanized MAbs effectively prevent adhesion of human T cells to human keratinocytes and the proliferation of T cells in response to nonautologous leukocytes in the mixed lymphocytes response (MLR), a model for responsiveness to MHC class II antigens (McCabe *et al., Cellular Immunol.* 150:364-375 (1993)). However, both the murine (Reimann *et al., Cytometry*, 17:102-108 (1994)) and humanized MAbs did not cross-react with nonhuman primate CD11a other than chimpanzee CD11a. In order to have a humanized MAb available for preclinical studies in rhesus, the humanized MAb was re-engineered to bind to rhesus CD11a by changing four residues in one of the complementarity-determining regions, CDR-H2, in the variable heavy domain. Cloning and molecular modeling of the rhesus CD11a I-domain suggested that a change from a lysine residue in human CD11a I-domain to glutamic acid in rhesus CD11a I-domain is the reason that the murine and humanized MAbs do not bind rhesus CD11a.

Materials and Methods

(a) Construction of humanized F(ab')s

The murine anti-human CD11a MAb, MHM24 (Hildreth *et al., Eur. J. Immunol.* 13:202-208 (1983); Hildreth *et al., J. Immunol.* 134:3272-3280 (1985)), was cloned and sequenced. In order to have a plasmid useful for mutagenesis as well as for expression of F(ab)s in *E. coli*, the phagemid pEMX1 was constructed. Based on the phagemid pb0720, a derivative of pB0475 (Cunningham *et al., Science* 243:1330-1336 (1989)), pEMX1 contains a DNA fragment encoding a humanized κ-subgroup I light chain and a humanized subgroup III heavy chain (VH-CH1) and an alkaline phosphatase promotor and Shine-Dalgarno sequence both derived from another previously described pUC119-based plasmid, pAK2 (Carter *et al., Proc. Natl. Acad. Sci. USA* 89:4285 (1992)). A unique SpeI restriction site was also introduced between the DNA encoding for the F(ab) light and heavy chains.

To construct the first F(ab) variant of humanized MHM24, F(ab)-1, site-directed mutagenesis (Kunkel, *Proc. Natl. Acad. Sci. USA* 82:488 (1985)) was performed on a deoxyuridine-containing template of pEMX1; all six CDRs were changed to the MHM24 sequence. All other F(ab) variants were constructed from a template of F(ab)-1. Plasmids were transformed into *E. coli* strain XL-1 Blue (Stratagene, San Diego, CA) for preparation of double- and single-stranded DNA. For each variant both light and heavy chains were completely

sequenced using the dideoxynucleotide method (Sequenase. U.S. Biochemical Corp.). Plasmids were transformed into *E. coli* strain 16C9, a derivative of MM294, plated onto LB plates containing 5 μg/ml carbenicillin, and a single colony selected for protein expression. The single colony was grown in 5 ml LB-100 μg/ml carbenicillin for 5-8 h at 37° C. The 5 ml culture was added to 500 ml AP5-100 μg/ml carbenicillin and allowed to grow for 16 h in a 4 L baffled shake flask at 37° C. APS media consists of : 1.5 g glucose, 11.0 Hycase SF, 0.6 g yeast extract (certified), 0.19 g MgSO4 (anhydrous), 1.07 g NH4Cl, 3.73 g KCl, 1.2 g NaCl, 120 ml 1 M triethanolamine, pH 7.4, to 1 L water and then sterile filtered through 0.1 μm Sealkeen filter.

Cells were harvested by centrifugation in a 1 L centrifuge bottle (Nalgene) at 3000 x *g* and the supernatant removed. After freezing for 1 h, the pellet was resuspended in 25 ml cold 10 mM MES-10 mM EDTA, pH 5.0 (buffer A). 250 μl of 0.1 M PMSF (Sigma) was added to inhibit proteolysis and 3.5 ml of stock 10 mg/ml hen egg white lysozyme (Sigma) was added to aid lysis of the bacterial cell wall. After gentle shaking on ice for 1 h, the sample was centrifuged at 40,000 x *g* for 15 min. The supernatant was brought to 50 ml with buffer A and loaded onto a 2 ml DEAE column equilibrated with buffer A. The flow-through was then applied to a protein G-Sepharose CL-4B (Pharmacia) column (0.5 ml bed volume) equilibrated with buffer A. The column was washed with 10 ml buffer A and eluted with 3 ml 0.3 M glycine, pH 3.0, into 1.25 ml 1 M Tris, pH 8.0. The F(ab) was then buffer exchanged into PBS using a Centricon-30 (Amicon) and concentrated to a final volume of 0.5 ml. SDS-PAGE gels of all F(ab)s were run to ascertain purity and the molecular weight of each variant was verified by electrospray mass spectrometry.

(b) Construction of chimeric and humanized IgG

For generation of human IgG1 variants of chimeric (chIgG1) and humanized (HuIgG1) MHM24, the appropriate murine or humanized variable light and variable heavy (F(ab)-8, Table II) domains were subcloned into separate previously described pRK vectors (Gorman *et al., DNA Protein Eng. Tech.* 2:3 (1990)). Alanine-scan variants were constructed by site-directed mutagenesis (Kunkel, *Proc. Natl. Acad. Sci. USA* 82:488 (1985)) of the HuIgG1 light and heavy chain plasmids. The DNA sequence of each variant was verified by dideoxynucleotide sequencing.

Heavy and light chain plasmids were cotransfected into an adenovirus-transformed human embryonic kidney cell line, 293 (Graham *et al., J. Gen. Virol.* 36:59 (1977)), using a high efficiency procedure (Graham *et al., J. Gen. Virol.* 36:59 (1977); Gorman *et al., Science*, 221:551 (1983)). Media was changed to serum-free and harvested daily for up to 5 days. Antibodies were purified from the pooled supernatants using protein A-Sepharose CL-4B (Pharmacia). The eluted antibody was buffer exchanged into PBS using a Centricon-30 (Amicon), concentrated to 0.5 ml, sterile filtered using a Millex-GV (Millipore) and stored at 4° C.

The concentration of antibody was determined using total Ig-binding ELISA. The concentration of the reference humanized anti-p185^{HER}IgG1 (Carter *et al., Proc. Natl. Acad. Sci. USA* 89:4285 (1992)) was determined by amino acid composition analysis. Each well of a 96-well plate was coated with 1 μg/ml of goat anti-human IgG F(ab')$_2$ (Cappel Laboratories, Westchester, PA) for 24 h at 4° C. Purified antibodies were diluted and added in duplicate to the coated plates. After 1.5 h incubation, the plates were washed with PBS-0.02% Tween 20 and 0.1 ml of a 1:2000 dilution of horseradish peroxidase-conjugated sheep anti-human IgG F(ab')$_2$ (Cappel) was added. After 1.5 h incubation the plates were washed and 0.1 ml 0.2 mg/ml o-

phenylenediamine dihydrochloride-0.01% hydrogen peroxide-PBS was added. After 10 min. the reaction was stopped with 2 M sulfuric acid and the O.D. 490 nm was read.

(c) Cloning of rhesus CD11a I-domain

The DNA sequence of the rhesus I-domain was obtained using RT-PCR and primers derived from the human CD11a DNA sequence. Briefly, mRNA was isolated from ~10^7 rhesus leukocytes using the Fast Track mRNA purification kit (Invitrogen). 10 μg mRNA was reverse transcribed using MuLV reverse transcriptase. The first strand cDNA was then amplified by 40 cycles of PCR using the primers:
5'-CACTTTGGATACCGCGTCCTGCAGGT-3' (forward) (SEQ ID NO:21) and
5'-CATCCTGCAGGTCTGCCTTCAGGTCA-3' (reverse) (SEQ ID NO:22).

A single band of the predicted size was purified from the PCR reaction using agarose gel electrophoresis. The PCR product was digested with restriction enzyme Sse8387I (Takara) and ligated to a human CD11a-containing plasmid digested with the same restriction enzyme. There are two Sse8387I sites in the human CD11a sequence, one on either side of the I-domain. The resulting plasmid encoded a chimera consisting of human CD11a with a rhesus I-domain substituted for the human I-domain. DNA sequence analysis revealed five amino acid differences between human and rhesus. One difference was in the region N-terminal to the I-domain (Thr59Ser) and the other four differences were in the I-domain itself: Val133Ile, Arg189Gln, Lys197Glu, and Val308Ala (Fig. 2).

(d) FACScan analysis of F(ab) and IgG binding to Jurkat cells

Aliquots of 10^6 Jurkat T-cells were incubated with serial dilutions of humanized and control antibodies in PBS-0.1% BSA-10 mM sodium azide for 45 min at 4°C. The cells were washed and then incubated in fluorescein-conjugated goat anti-human $F(ab')_2$ (Organon Teknika, Westchester, PA) for 45 min at 4°C. Cells were washed and analysed on a FACScan (Becton Dickinson, Mountain View, CA). 8 x 10^3 cells were acquired by list mode and gated by forward light scatter versus side light scatter, thereby excluding dead cells and debris.

(e) Saturation binding to determine apparent K_ds

Radiolabeled antibodies were prepared using Iodo-Gen (Pierce, Rockford, IL) according to the manufacturer's instructions. 50 μg of antibody and 1 mCi ^{125}I (DuPont, Wilmington, DE) were added to each tube and incubated for 15 min at 25°C. Radiolabeled proteins were purified from the remaining free ^{125}I using PD-10 columns (Pharmacia, Uppsala, Sweden) equilibrated in Hank's Balanced Salt Solution (HBSS, Life Technologies, Grand Island, NY) containing 0.2% gelatin.

Mononuclear cells were purified from heparinized human peripheral blood collected from two donors using Lymphocyte Separation Medium (LSM, Organon Teknika, Durham, NC) according to the manufacturer's instructions. The blood was centrifuged at 400 x *g* for 40 min at 25°C with no braking. Cells at the interface of the LSM and plasma were harvested and then resuspended in HBSS-0.2% gelatin.

Leukocytes were purified from heparinized rhesus monkey peripheral blood collected from two individuals by Dextran sedimentation. Blood was diluted with an equal volume of 3% Dextran T500 (Pharmacia) in PBS and was allowed to sediment undisturbed at 25°C for 30 min. After sedimentation, cells remaining in suspension were harvested and pelleted by centrifugation at 400 x *g* for 5 min. Residual

erythrocytes were removed by two cycles of hypotonic lysis using distilled water and 2X HBSS. After erythrocyte lysis, cells were washed in PBS and then resuspended in HBSS-0.2% gelatin.

Antibody affinities were determined by saturation binding using either peripheral blood mononuclear cells (murine MHM24 and HuIgG1) or rhesus leukocytes (MHM23, RhIgG1). In each assay, a radiolabeled antibody was serially diluted in HBSS-0.2% gelatin in quadruplicate. Nonspecific binding was determined by the addition of 500nM final concentration of homologous unlabeled antibody in duplicate through the serial dilution. Human lymphocytes or rhesus leukocytes were added to the plates in a volume of 170 µl per well. Plates were incubated for 2 hr at room temperature on an orbital plate shaker. After incubation, cells were harvested using a SKATRON™ cell harvester (Lier, Norway) and washed 10 times with PBS containing 0.25% gelatin and 0.1% sodium azide. Samples were then counted for 1 min in an LBK Wallac GammaMaster gamma counter (Gaithersburg, MD). Data was transformed from counts per minute to nanomolarity and four-parameter curve-fitting of saturation plots (bound versus total) was then performed to determine K_d (app) values.

(f) Keratinocyte monolayer adhesion assay

Normal human epidermal keratinocytes (Clonetics, San Diego, CA) were removed from culture flasks with trypsin-EDTA, centrifuged, and resuspended in lymphocyte assay medium (RPMI 1640 (GIBCO)-10% fetal calf serum-1% penicillin/streptomycin) at a concentration of 5×10^5 viable cells/ml. Aliquots of 0.1 ml/well were then cultured overnight in flat-bottom 96-well plates; appropriate wells were stimulated by addition of interferon-gamma (Genentech, South San Francisco, CA) at 100 units/well.

Jurkat clone E6-1 cells (ATCC, Rockville, MD) or purified rhesus lymphocytes (see MLR methods) were labeled with 20 µg/ml Calcein AM (Molecular Probes, Eugene, OR) at 37° C for 45 min. After washing three times with lymphocyte assay medium, Jurkat or rhesus lymphocyte cells were resuspended to 1×10^6 cells/ml and incubated with serially-diluted antibody at 4° C for 30 min. After removal of medium from the keratinocyte monolayer, 0.1 ml/well of labeled cells were added and incubated at 37° C for 1 h. The wells were washed five times with 0.2 ml/well/wash of 37° C lymphocyte medium to remove non-attached cells. Fluorescence was measured using a Cytofluor 2300 (Millipore, Bedford, MA).

A rhesus-human chimeric CD11a (Rh/HuCD11a) comprising human CD11a with a rhesus I-domain was constructed by site-directed mutagenesis (Kunkel, *Proc. Natl. Acad. Sci. USA* 82:488 (1985)) on a deoxyuridine-containing template plasmid encoding human CD11a. Four residues were altered: Val133Ile, Arg189Gln, Lys197Glu, and Val308Ala (Fig. 2). Plasmids coding for Rh/HuCD11a and human CD11b (EP 364, 690) were cotransfected into an adenovirus-transformed human embryonic kidney cell line, 293 (Graham *et al., J. Gen. Virol.* 36:59 (1977)), using a high efficiency procedure (Graham *et al., J. Gen. Virol.* 36:59 (1977); Gorman *et al., Science*, 221:551 (1983)). Rh/HuCD11a-transfected 293 cells were labeled with 20 µg/ml Calcein AM at 37°C for 45 min. After washing three times with lymphocyte assay medium, Rh/HuCD11a-transfected 293 cells were resuspended to 1×10^6 cells/ml and incubated with serially-diluted antibody at 4° C for 30 min. After removal of medium from the keratinocyte monolayer, 0.1 ml/well of labeled 293 cells was added and incubated at 37° C for 1 h. The wells were washed five times with 0.2 ml/well/wash of 37° C lymphocyte medium to remove non-attached cells. Fluorescence was measured using a Cytofluor 2300.

(g) ICAM adhesion assay

Maxisorp (Nunc) 96-well plates were coated with 0.1 ml/well of 1 μg/ml goat anti-human IgG Fc (Caltag) for 1 h at 37°C. After washing the plates three times with PBS, the plates were blocked with 1% BSA-PBS for 1 h at 25°C. The plates were then washed three times with PBS and 0.1 ml/well of 50 ng/ml recombinant human ICAM-IgG was added and incubated overnight.

The ICAM-IgG consisted of the five extracellular domains of human ICAM fused onto a human IgG Fc. A plasmid for the expression of a human ICAM-1 (Simmons *et al. Cell* 331:624-627 (1988) and Staunton *et al. Cell* 52:925-933 (1988)) immunoadhesin called pRK.5dICAMGaIg was constructed. It contains the five Ig-like domains of ICAM-1, a six amino acid cleavage site recognized by an H64A variant of subtilisin BPN', Genenase I (Carter *et al. Proteins: Structure, Function and Genetics* 6:240-248 (1989)), and the Fc region from human IgG1 (Ellison *et al. Nucleic Acids Research* 10:4071-4079 (1982)) in the pRK5 vector (Eaton *et al. Biochemistry* 25:8343-8347 (1986)). Human embryonic kidney 293 cells (Graham *et al. J. Gen. Virol.* 36:59 (1977)) were stably transfected with pRK.5dICAMGaIg and the RSV-neo plasmid (Gorman *et al. Science* 221:551-553 (1983)) to generate a cell line expressing the five domain ICAM Ig (5dICAMIg). A clone was selected which expressed 20 μg/ml of secreted 5dICAMIg by enzyme-linked immunosorbent assay (ELISA), using antibodies to human IgG Fc (Caltag, Burlingame, CA) and ICAM-1 (BBIG-I1; R & D Systems, Minneapolis, MN). Cell culture supernatant from this cell line was loaded onto a Protein A column (ProsepA, Bioprocessing, Ltd., Durham, England) equilibrated in 0.01 M Hepes buffer (pH 7.0), 0.15 M NaCl (HBS) and the column was washed with HBS followed by 0.01 M Hepes buffer (pH 7.0), 0.5 M NaCl, 0.5 M TMAC (tetra-methyl ammonium chloride) to remove non-specifically bound material. The TMAC buffer was thoroughly washed from the column with HBS and the 5dICAMIg eluted with 0.01 M Hepes buffer (pH 7.0), 3.5 M MgCl2 and 10% (w/v) glycerol. The protein A pool was dialyzed extensively against HBS and concentrated.

Purified rhesus lymphocytes (see MLR methods) were labeled with 20 μg/ml Calcein AM (Molecular Probes, Eugene, OR) at 37°C for 45 min. After washing three times with lymphocyte assay medium, rhesus lymphocyte cells were resuspended to 1 x 10^6 cells/ml and incubated with serially-diluted antibody at 4°C for 30 min. After removal of medium from the ICAM-IgG coated plates, 0.1 ml/well of labeled cells were added and incubated at 37°C for 1 h. The wells were washed five times with 0.2 ml/well/wash of 37°C lymphocyte medium to remove non-attached cells. Fluorescence was measured using a Cytofluor 2300 (Millipore, Bedford, MA).

(h) One-way mixed lymphocyte response (MLR)

For both human and rhesus MLR, peripheral blood lymphocytes from two unrelated donors were isolated from whole, heparinized blood using Lymphocyte Separation Medium (Organon Teknika, Durham, NC). Lymphocytes were resuspended to a concentration of 3 x 10^6 cells/ml in RPMI 1640 (GIBCO)-10% human AB serum-1% glutamine-1% penicillin/streptomycin-1% non-essential amino acids-1% pyruvate-5 x 10^{-5} M 2-β-mercaptoethanol-50 μg/ml gentamycin-5 μg/ml polymyxin B. The stimulator cells were made unresponsive by irradiation with 3000 rads in a cesium irradiator. Responder cells at a concentration of 1.5 x 10^5 cells per well were co-cultured with an equal number of stimulator cells in 96-well, flat-bottom plates. Serial two-fold dilutions of each antibody were added to the cultures to give a total volume of 200 μl/well. The

cultures were incubated at 37°C in 5% CO2 for 5 days and then pulsed with 1 μCi/well of [^{3}H]thymidine for 16 h. [^{3}H]thymidine incorporation was measured with a Beckman scintillation counter. Assays were done in triplicate. A humanized anti-human p185^{HER2} MAb (Carter *et al., Proc. Natl. Acad. Sci. USA* 89:4285 (1992)) was used as isotype control for HuIgG1 and RhIgG1. A murine anti-hamster tPA MAb (Genentech) was used as isotype control (murine IgG1) for the MHM23 MAb. MAb 25.3 was purchased from Immunotech, Inc. (Westbrook, ME).

(i) Computer graphics models of murine and humanized MHM24

Sequences of the VL and VH domains (Figs. 1A & B) were used to construct a computer graphics model of the murine MHM24 VL-VH domains. This model was used to determine which framework residues should be incorporated into the humanized antibody. A model of F(ab)-8 was also constructed to verify correct selection of murine framework residues. Construction of the models was performed as described previously (Carter *et al., Proc. Natl. Acad. Sci. USA* 89:4285 (1992); Eigenbrot *et al., J. Mol. Biol.* 229:969 (1993)).

Results

(a) Humanization

The consensus sequence for the human heavy chain subgroup III and the light chain subgroup κ I were used as the framework for the humanization (Kabat *et al., Sequences of Proteins of Immunological Interest.* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) (Figs. 1A & B). This framework has been successfully used in the humanization of other murine antibodies (Carter *et al., Proc. Natl. Acad. Sci. USA* 89:4285 (1992); Presta *et al., J. Immunol.* 151:2623-2632 (1993); Eigenbrot *et al., Proteins* 18:49-62 (1994)). All humanized variants were initially made and screened for binding as F(ab)s expressed in *E. coli.* Typical yields from 500 ml shake flasks were 0.2-0.5 mg F(ab). Mass spectrometry verified the mass of each F(ab) to within 5 mass units.

CDR-H1 included residues H28-H35, which includes all exposed residues from both Kabat *et al., Sequences of Proteins of Immunological Interest.* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991) and Chothia *et al. Nature* 342:877-883 (1989). The other hypervariable loops were defined according to Chothia *et al.* (1989). Light chain residue numbers are prefixed with L; heavy chain residue numbers are prefixed with H.

Table II

Binding of humanized MHM24 variants to human CD11a on Jurkat cells

				EC50 F(ab)/ EC50 F(ab)-2[b]		
Variant	Template	Changes[a]	Purpose	Mean	S.D.	N
F(ab)-1	Human FR	ArgH71**Val**	Straight CDR swap	no binding		2
F(ab)-2	F(ab)-1	AlaH60**Asn** AspH61**Gln** SerH62**Lys** ValH63**Phe** GlyH65**Asp**	Extended CDR-H2 (Kabat *et al.* (1991))	1.0		4
F(ab)-3	F(ab)-2	PheH67**Ala**	Packing;CDR-H2	1.2	0.33	3
F(ab)-4	F(ab)-2	ValH71**Arg**	Packing;CDR-H1,H2	2.9		1
F(ab)-5	F(ab)-2	AsnH73**Lys** LysH75**Ser** AsnH76**Ser**	Framework loop in VH	0.043	0.015	4
F(ab)-6	F(ab)-5	SerL53**Thr** GluL55**Gln**	Extended CDR-L2	0.012	0.005	4
F(ab)-7	F(ab)-6	PheH27**Tyr**	Extended CDR-H1	0.004	0.002	4
F(ab)-8	F(ab)-7	SerH75Lys SerH76Asn	Framework loop in VH back to human	0.004	0.002	4
HuIgG1[c]				0.004	0.002	4
chIgG1[d]				0.006	0.005	4

[a] Murine residues are in bold; residue numbers are according to Kabat *et al., Sequences of Proteins of Immunological Interest* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991).
[b] Mean and standard deviation are the average of the ratios calculated for each of the independent FACScan assays; the EC50 for F(ab)-2 was 771 ± 320 ng/ml.
[c] HuIgG1 is F(ab)-8 VL and VH domains fused to human constant light and heavy chains.
[d] chIgG1 is chimeric IgG with murine VL and VH domains fused to human constant light and heavy chains.

In the initial variant F(ab)-1 the CDR residues were transferred from the murine antibody to the human framework. In addition, residue H71 was changed from the human Arg to murine Val since this residue has been shown previously to affect the conformations of CDR-H1 and CDR-H2 (Chothia *et al., Nature*, 342:877-883 (1989); Tramontano *J. Mol. Biol.* 215:175 (1990)). This F(ab) showed no detectable binding. In F(ab)-2 CDR-H2 was extended to include the sequence-based definition (*i.e.*, including residues H60-H65). The EC50 for F(ab)-2 binding to human CD11a was 771 ± 320 ng/ml, which was 148-fold weaker than the EC50 of the chimeric IgG1 (5.2 ± 3.0 ng/ml).

In previous humanizations, it has been found that residues in a framework loop (FR-3) adjacent to CDR-H1 and CDR-H2 can affect binding (Eigenbrot *et al., Proteins* 18:49-62 (1994)). In F(ab)-5 three residues in this loop were changed to their murine counterpart and this variant showed a 23-fold improvement in binding (Table II). Alteration of human residues at positions L53 and L55 to murine (*i.e.* SerL53Thr and GluL55Gln) further improved binding by another 4-fold (F(ab)-6, Table II); this effectively converted CDR-L2 from the structure-based definition (residues L50-L52) to the sequence-based definition (residues L50-L56). Subsequent alteration of PheH27 to murine Tyr in CDR-H1 resulted in an additional 3-fold improvement (F(ab)-7; Table II). Finally, based on the models of murine and humanized MHM24, two of the three murine residues (H75 and H76) in FR-3 were changed back to human and it was found that these two residues had no effect on binding (cf. F(ab)-7 and F(ab)-8, Table II). The average EC50 for F(ab)-8 was slightly better than that of the chimeric IgG1 (Table II). Not all changes from human to murine resulted in improved binding. PheH67 was changed to murine Ala since this position had been previously found to affect binding (Presta *et al., J. Immunol.* 151:2623-2632 (1993)) but no effect was evident (F(ab)-3, Table II). Changing ValH71 back to the human Arg effected a 3-fold reduction in binding (F(ab)-4, Table II), supporting inclusion of ValH71 in F(ab)-1.

The VL and VH domains from F(ab)-8 were transferred to human IgG1 constant domains. The full length intact antibody, HuIgG1, showed an EC50 equivalent to F(ab)-8 and improved compared to the full length chimeric IgG1 (Table II). When data for all assays of HuIgG1 is considered, including its use as a standard for the alanine-scan and MLR assays (see below), the EC50 for HuIgG1 against human CD11a was 0.042 ± 0.072 nM (N = 15). Saturation binding analysis was also performed to determine the apparent dissociation constants, K_d(app): 0.15 ± 0.02 nM for murine MHM24 and 0.15 ± 0.04 nM for HuIgG1 (Table III).

Table III

Apparent K_d by saturation binding to human lymphocytes and rhesus leukocytes

	muMHM24 K_d(app) nM	HuIgG1 K_d(app) nM	muMHM23 K_d(app) nM	RhIgG1 K_d(app) nM
human donor 1	0.16 +/- 0.01	0.11 +/- 0.08		
human donor 2	0.13 +/- 0.02	0.18 +/- 0.03		
rhesus donor 1			3.9 +/- 0.31	3.9 +/- 1.04
rhesus donor 2			4.5 +/- 0.51	n.d.
rhesus donor 3				2.8 +/- 1.1[a]
rhesus donor 3				2.7 +/- 0.9

[a] Assays of rhesus donor 3 were performed using two batches of RhIgG1; the assays were performed in the presence of 1 mg/ml human IgG1 to block Fc receptor interaction.

(b) Alanine-Scan of CDR Residues

To determine which CDR residues were involved in binding to human CD11a an alanine-scan (Cunningham *et al., Science* 244:1081 (1989)) was performed on the CDR residues of HuIgG1. Each variant was tested for binding to CD11a on Jurkat cells. In the light chain only CDR-L3 contributes to binding. HisL91 had a large effect (Table IV) and is probably conformational since this side chain should be partially buried. Residues AsnL92 and TyrL94 had more modest effect, reducing binding by 3-fold and 12-fold, respectively. Note however that simultaneously changing these two residues to alanine (as well as GluL93Ala) had a non-additive effect on binding (variant L3, Table IV).

Table IV

Alanine-scan of humanized MHM24 CDR residues

Variant IgG1		Human CD11a Var.EC50/HuIgG1 EC50[b]			Rhesus CD11a Var.EC50/HuIgG1 EC50[c]		
CDR Sequence[a]		Mean	S.D	N	Mean	S.D.	N
CDR-H1	G Y S F T G H W M N						
H1[d]	A A A	5.9	0.8	2	nb		
SerH28Ala	A	6.9	0.1	2	nb		
ThrH30Ala	A	1.7	0.3	2	1.3		
GlyH31Ala	A	1.2	0.1	2	2.4		
HisH32Ala	A	2.3	0.2	2	nb		
TrpH33Ala	A	>870		1	nb		
CDR-H2	M I H P S D S E T R Y N Q K F K D						
H2	A A A	14.1	7.8	10	0.055	0.050	15
H2B	A A A	>600		2	nb		
H2A1	A A	10.8	7.3	6	0.013	0.012	10
H2A2	A A	1.9	0.1	2	1.1	0.1	2
H2A3	A A	4.6	0.2	2	1.3		
HisH52Ala	A	1.5	0.2	2	0.7		
HisH52Ser	S	5.0	0.3	2	nb		
SerH53Ala	A	1.8	0.1	2	0.7		
AspH54Ala	A	147	18	2	0.3		
SerH55Ala	A	1.3	0.1	2	1.3		
SerH55Asn	N	2.1	0.2	2	nb		
SerH55Gln	Q	2.9	1.4	2	6.7		
GluH56Ala	A	4.0	0.6	2	0.3		
ArgH58Ala	A	1.1	0.1	2	3.3		
GlnH61Ala	A	4.1	0.1	2	5.3		
LysH62Ala	A	1.8	0.2	2	4.9		
LysH64Ala	A	2.5	0.1	2	1.2		
AspH65Ala	A	0.8	0.1	2	1.1		
FR-3							
LysH73Ala		5.2	0.9	2	nb		
LysH73Arg		4.6	1.1	2	5.5		
CDR-H3	G I Y F Y G T T Y F D						
H3	A A	>900		2	nb		
H3B	A A A	34.7	13.6	2	nb		
TyrH97Ala	A	10.9	2.1	2	nb		
TyrH99Ala	A	1.4	0.1	2	nb		
ThrH100aAla	A	2.3	0.6	2	nb		

Variant IgG1		Human CD11a Var.EC50/HuIgG1 EC50[b]			Rhesus CD11a Var.EC50/HuIgG1 EC50[c]		
CDR Sequence[a]		Mean	S.D	N	Mean	S.D.	N
ThrH100bAla	A	1.4	0.2	2	nb		
TyrH100cAla	A	7.6	1.0	2	nb		
CDR-L1	RASKTISKYLA						
L1	AA AA	1.3	0.3	3	1.0		
CDR-L2	SGSTLQS						
L2	A AA	1.1	0.0	2	nb		
SerL50Ala	A	1.2	0.5	2	2.7		
SerL52Ala	A	1.1	0.2	2	13.3		
ThrL53Ala	A	0.9	0.4	2	0.7		
CDR-L3	QQHNEYPLT						
L3	AAA	>900		2	nb		
HisL91Ala	A	>900		2	nb		
AsnL92Ala	A	3.3	0.7	2	3.3		
GluL93Ala	A	1.7	0.2	2	2.9		
TyrL94Ala	A	11.8	0.1	2	nb		
hu4D5[e]		>900		5	nb		

[a] CDRs and FR-3 are as defined in Kabat *et al.*, (1991) *supra.*

[b] EC50 HuIgG1 for human CD11a = 0.042 nM (S.D.= 0.072; N = 15).

[c] EC50 HuIgG1 for rhesus CD11a = 45.6 nM (S.D.= 40.4; N = 16); all values for rhesus CD11a are for a single binding assay unless otherwise noted; nb denotes binding of variant is greater than 10-fold weaker than HuIgG1.

[d] Multiple alanine variants:
H1, SerH28Ala/ThrH30Ala/HisH32Ala;
H2,HisH52Ala/SerH53Ala/SerH55Ala;
H2B, AspH54Ala/GluH56Ala/ArgH58Ala;
H2A1, HisH52Ala/SerH53Ala;
H2A2, SerH53Ala/SerH55Ala;
H2A3, HisH52Ala/SerH55Ala;
H3,TyrH97Ala/TyrH99Ala;
H3B, ThrH100aAla/ThrH100bAla/TyrH100cAla;
L1, LysL27Ala/ThrL28Ala/SerL30Ala/LysL31Ala;
L2, SerL50Ala/SerL52Ala/ThrL53Ala;
L3 AsnL92Ala/GluL93Ala/TyrL94Ala.

[e] hu4D5 is a humanized anti-p185^{HER2} antibody with the same IgG1 framework as the huMHM24 antibody (Carter *et al., Proc.Natl. Acad. Sci. USA* 89:4285 (1992)).

In the heavy chain, CDR-H2 and CDR-H3 are the prominent contributors to the binding. CDR-H1 residue TrpH33Ala had a large effect but this is most likely due to a conformational change as TrpH33 should be partially buried. The most important single residue contributing to the binding is AspH54 in CDR-H2; changing this residue to alanine effected a 147-fold reduction in binding (Table IV). Other residues in CDR-H2 involved in binding include GluH56, GlnH61 and LysH64 (Table IV). In CDR-H3, TyrH97Ala reduced binding by 11-fold and TyrH100cAla by 8-fold. As in CDR-L3, simultaneous alteration of several CDR-H3 residues to alanine effected a non-additive, large reduction in binding (cf. variant H3 versus TyrH97Ala and

TyrH99Ala, Table IV). In addition, the FR-3 residue included in the humanization, LysH73, also showed a 5-fold reduction in binding when changed to alanine or arginine (Table IV).

(c) Re-engineering HuIgG1 to Bind to Rhesus CD11a

Both murine MHM24 and HuIgG1 showed approximately 1000-fold reduction in binding to rhesus CD11a: HuIgG1 had an EC50 against rhesus CD11a of 45.6 ± 40.4 nM (N = 16) compared to an EC50 of 0.042 ± 0.072 nM against human CD11a. Since a primate model is important in evaluating the biology, toxicity, and efficacy of MHM24, improving the binding of HuIgG1 to rhesus CD11a was deemed advantageous. Initially, the MAb hypervariable region residues which were important in binding to human CD11a and rhesus CD11a were determined so that those important for the rhesus but not the human could be altered. Accordingly, the alanine-scan variants were also assayed against rhesus CD11a on peripheral blood lymphocytes. The most important finding was that one of the multiple-alanine mutation variants, variant H2, bound 18-fold better to rhesus CD11a than HuIgG1 (Table IV). However, individual mutations at the three residues included in variant H2 showed minimal improvement in binding: HisH52Ala, 0.7-fold better, SerH53Ala, 0.7-fold better, and SerH55Ala, 1.3-fold worse (Table IV). A series of double mutations at these three residues showed that the combination HisH52Ala-SerH53Ala was the best, providing a 77-fold improvement in binding compared to HuIgG1 (cf. variants H2A1, H2A2 and H2A3, Table IV). In addition, the AspH54Ala and GluH56Ala variants also effected a 3-fold improvement over HuIgG1 (Table IV), even though AspH54 is the most important binding residue in HuIgG1 with respect to human CD11a.

In an attempt to find a single substitution at position H54 which would improve binding to rhesus CD11a but not reduce binding to human CD11a, position H54 was substituted with a variety of amino acids. All substitutions reduced binding by greater than an order of magnitude whereas the substitution AspH54Asn improved rhesus binding by 10-fold (Table V).

Table V
Amino acid substitution at AspH54

AspH54 change to	Variant EC50/HuIgG1 EC50[a]		
	Human CD11a[b]		Rhesus CD11a[c]
	mean	S.D.	
Ala	147	18	0.3
Asn	26	1	0.1
Gln	20	1	4.4
Glu	16	2	>25
Ser	>100		0.9
Arg	>250		>25
Lys	>100		3.8
His	>300		>25
Thr	>450		>25

Met	>150		>25
Leu	>300		>25

[a] EC50 HuIgG1 for human CD11a = 0.042 nM (S.D.= 0.072; N=15); EC50 HuIgG1 for rhesus CD11a = 45.6 nM (S.D.= 40.4; N=16).
[b] Values are the mean of two assays.
[c] Values are for a single assay.

Since non-additive effects were noted for changes at positions H52-H53, these were combined with a variety of changes at positions H54 and H56 (Table VI). For all variants, H52 and H53 were alanine. In one series, position H54 was Asn and position H56 was Glu (original), Ala, Asn or Gln. None of these variants improved rhesus CD11a binding over the H2A1 variant (Table VI). In another series, position H54 was Ala and position H56 was Glu (original), Ala, Ser or Asn and again all were worse than variant H2A1. In the third series, position H54 was Ser and position H56 was Glu (original), Ala, Ser or Asn. Two of these variants exhibited improved binding to rhesus CD11a compared to the H2A1 variant (H2C11 and H2C12, Table VI). The rhesus CD11a EC50 for these two variants was 0.11 ± 0.11 nM (N = 9) for H2C11 and 0.19 ± 0.08 nM (N = 7) for H2C12. These values are 3- to 5-fold weaker than the EC50 of HuIgG1 for human CD11a (0.042 nM) but are a 240- to 415-fold improvement over the EC50 of HuIgG1 for rhesus CD11a (45.6 nM). H2C12 will hereafter be referred to as RhIgG1. Apparent K_d values from saturation binding experiments showed that RhIgG1 bound to rhesus CD11a as well as murine MHM23 bound to rhesus CD18 (Table III).

Table VI

Binding of CDR-H2 variants to human and rhesus CD11a

Variant IgG1	Sequence	Var.EC50/HuIgG1 EC50[a]		
		Human CD11a	Rhesus CD11a	
			Mean	S.D.
H2C2	A	2.6	>100	
H2C3	A A A N	>100	>100	
CDR-H2	M I H P S D S E T R Y	1.0	1.00	
H2A1	A A	10.8	0.013	0.012(N=10)
H2C1	A . A N	>100	0.56	0.01
H2C4	A A N A	>100	0.38	0.06
H2C5	A A N N	46	0.11	0.02
H2C6	A A N Q	>100	0.21	0.01
H2C8	A . A A	12.7	0.38	
H2C7	A A A A	2.4	1.03	0.05
H2C10	A A A S	14.2	0.22	0.03

H2C9	A	A A	N	34.3	0.22	0.04
H2C13	A	A S			0.10	0.06
H2C14	A	A S	A		0.021	0.013
H2C12	A	A S	S		0.004	0.001 (N=7)
H2C11	A	A S	N	24.9	0.002	0.001(N=9)

[a] EC50 HuIgG1 for human CD11a = 0.042 nM (S.D.= 0.072; N = 15); EC50 HuIgG1 for rhesus CD11a = 45.6 nM (S.D.= 40.4; N = 16); all values for rhesus CD11a are the mean of two independent binding assays except as noted.

For the HuIgG1-human CD11a interaction, AspH54 was the most important residue (Table IV); changing this residue to other amino acids significantly reduced binding with the least reduction occurring for changes to Glu, Asn and Gln. However, for the HuIgG1-rhesus CD11a interaction, AspH54 was deleterious since changing this residue to Ala or Asn improved binding (Table V). In order to understand this difference between binding to human and rhesus CD11a, the latter was cloned from a rhesus PBL library. Fig. 2 shows that rhesus CD11a I-domain differs from human CD11a I-domain at only four positions: 133, 189, 197, 308. Previously the human CD11a epitope of MHM24 was mapped to residues 197-203 (Champe *et al., J. Biol. Chem.* 270:1388-1394 (1995)) which includes the human Lys197 to rhesus Glu197 change in rhesus.

(d) Keratinocyte Cell Adhesion Assays

Murine MHM24, chimeric IgG1 and HuIgG1 were compared in their ability to prevent adhesion of Jurkat cells (human T-cells expressing LFA-1) to normal human epidermal keratinocytes expressing ICAM-1. All three antibodies performed similarly (Fig. 3) with similar IC50 values (Table VII).

Table VII

Blocking of cell adhesion by MHM24 variants

	IC50 Value (nM)			
mAb	Jurkat:HuK[a]	RhLy[b]:HuK Mean S.D. N	RhLy:HuICAM[c] Mean S.D. N	Rh/HuCD11a[d]:HuK Mean S.D. N
murMHM24	0.09			
HuIgG1	0.13			
chIgG1	0.10			
RhIgG1		119 86 4	5.3 4.5 3	4.9 0.2 2
MHM23		1.6 1.5 3	1.2	1.4 0.1 2

[a] HuK = normal human epidermal keratinocyte.
[b] RhLy = rhesus lymphocyte.
[c] HuICAM = recombinant human ICAM-1.
[d] Rh/HuCD11a = human CD11a with rhesus I-domain transfected into human 293 cells.

Neither murine nor humanized MHM24 blocked rhesus or cynomolgus lymphocytes from adhering to human keratinocytes. When RhIgG1 was compared to the murine anti-human CD18 antibody MHM23

(Hildreth *et al., Eur. J. Immunol.* 13:202-208 (1983); Hildreth *et al., J. Immunol.* 134:3272-3280 (1985)) in blocking adherence of rhesus lymphocytes to human keratinocytes, RhIgG1 was 74-fold less efficacious than MHM23 (Fig. 4A, Table VII). However, when recombinant human ICAM-1 was coated on plates (instead of human keratinocytes) RhIgG1 was only 4-fold less efficacious than MHM23 (Fig. 4B, Table VII). A chimeric CD11a comprised of human CD11a in which the I-domain was mutated to rhesus (Val133Ile, Arg189Gln, Lys197Glu, Val308Ala) was transfected into human embryonic kidney 293 cells. Again, RhIgG1 was only 4-fold down from MHM23 in blocking these Rh/HuCD11a-293 cells from adhering to human keratinocytes (Fig. 4C, Table VII).

Control isotype antibodies for RhIgG1 (humanized anti-p185HER2 antibody; Carter *et al., Proc. Natl. Acad. Sci. USA* 89:4285 (1992)) and MHM23 (murine MAb 354, a murine IgG1 anti-hamster tPA) did not block binding of rhesus lymphocytes to human keratinocytes or recombinant ICAM-1 (Figs. 4A, 4B) or Rh/HuCD11a to human keratinocytes (Fig. 4C). This implies that the reduced performance of RhIgG1 compared to murine MHM23 in the rhesus lymphocyte:human keratinocyte assay was not due to any unexpected interaction of the human Fc of HuIgG1 (compared to the murine Fc of MHM23) with the rhesus lymphocytes, which might reduce the concentration of RhIgG1 available for binding to rhesus CD11a. The recombinant human ICAM-1 data show that RhIgG1 is binding to the rhesus lymphocytes and preventing adherence almost as well as murine MHM23 (Fig. 4B, Table VII). The Rh/HuCD11a-293 data (Fig. 4C, Table VII) show that RhIgG1 is not binding to targets on the human keratinocytes (compared to HuIgG1), which might reduce the concentration of RhIgG1 available for binding to rhesus CD11a. In addition, the K_d(app) of RhIgG1 to rhesus leukocytes was similar with (rhesus donor 3) and without (rhesus donor 1) addition of 1 mg/ml human IgG1 (Table III); This shows that binding of RhIgG1 is specific to rhesus CD11a.

(e) Mixed Lymphocyte Response Assays

In the MLR, HuIgG1 exhibited an IC50 value 2-fold weaker than the murine MHM24 (Table VIII, Fig. 5).

Table VIII
Mixed lymphocyte response assay results

	IC50 Value (nM)		
mAb[a]	Mean	S.D.	N
murMHM24	0.19	0.06	3
HuIgG1	0.38	0.14	4
mAb 25.3	3.8	1.0	2
RhIgG1	23.4	11.4	2
MHM23	30.4	24.0	3

[a] murMHM24, HuIgG1 and mAb 25.3 tested in human MLR; RhIgG1 and MHM23 tested in rhesus MLR.

Both the murine and humanized MAbs fared 10- to 20-fold better than MAb 25.3, which has been previously tested *in vivo* (Fischer *et al., Blood* 77:249-256 (1991); Stoppa *et al., Transplant Intl.* 4:3-7 (1991); Hourmant *et al., Transplantation* 58:377-380 (1994)). The rhesus-binding variant RhIgG1 exhibited an IC50 value slightly better than murine MHM23 (Table VIII). Different responder:stimulator blood donors were used

in independent assays and the results did not vary significantly. The K_d of RhIgG1 for rhesus CD11a is about 26-fold down from the K_d of HuIgG1 for human CD11a (Table III) and this is reflected in the IC50 values derived from the MLR assays (Table VIII).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Genentech, Inc.

(ii) TITLE OF INVENTION: Humanized Anti-CD11a Antibodies

(iii) NUMBER OF SEQUENCES: 24

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genentech, Inc.
(B) STREET: 1 DNA Way
(C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WinPatin (Genentech)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Lee, Wendy M.
(B) REGISTRATION NUMBER: 40,378
(C) REFERENCE/DOCKET NUMBER: P1014PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 650/225-1994
(B) TELEFAX: 650/952-9881

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 108 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Asp Val Gln Ile Thr Gln Ser Pro Ser Tyr Leu Ala Ala Ser Pro
 1               5                  10                  15

Gly Glu Thr Ile Ser Ile Asn Cys Arg Ala Ser Lys Thr Ile Ser
            20                  25                  30

Lys Tyr Leu Ala Trp Tyr Gln Glu Lys Pro Gly Lys Thr Asn Lys
        35                  40                  45

Leu Leu Ile Tyr Ser Gly Ser Thr Leu Gln Ser Gly Ile Pro Ser
    50                  55                  60
```

```
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                65                  70                  75

Ser Ser Leu Glu Pro Glu Asp Phe Ala Met Tyr Tyr Cys Gln Gln
                80                  85                  90

His Asn Glu Tyr Pro Leu Thr Phe Gly Thr Gly Thr Lys Leu Glu
                95                 100                 105

Leu Lys Arg
       108
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 108 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Lys Thr Ile Ser
            20                  25                  30

Lys Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
        35                  40                  45

Leu Leu Ile Tyr Ser Gly Ser Thr Leu Gln Ser Gly Val Pro Ser
    50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80                  85                  90

His Asn Glu Tyr Pro Leu Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                 100                 105

Ile Lys Arg
       108
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 108 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser
```

20 25 30

Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
35 40 45

Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
50 55 60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
65 70 75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
80 85 90

Tyr Asn Ser Leu Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
95 100 105

Ile Lys Arg
108

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 121 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Glu Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Met Arg Pro Gly
1 5 10 15

Ala Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Ser Phe Thr
20 25 30

Gly His Trp Met Asn Trp Val Arg Gln Arg Pro Gly Gln Gly Leu
35 40 45

Glu Trp Ile Gly Met Ile His Pro Ser Asp Ser Glu Thr Arg Leu
50 55 60

Asn Gln Lys Phe Lys Asp Lys Ala Thr Leu Thr Val Asp Lys Ser
65 70 75

Ser Ser Ser Ala Tyr Met Gln Leu Ser Ser Pro Thr Ser Glu Asp
80 85 90

Ser Ala Val Tyr Tyr Cys Ala Arg Gly Ile Tyr Phe Tyr Gly Thr
95 100 105

Thr Tyr Phe Asp Tyr Trp Gly Gln Gly Thr Thr Leu Thr Val Ser
110 115 120

Ser
121

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 121 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Thr
20 25 30

Gly His Trp Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45

Glu Trp Val Gly Met Ile His Pro Ser Asp Ser Glu Thr Arg Tyr
50 55 60

Asn Gln Lys Phe Lys Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
65 70 75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ala Arg Gly Ile Tyr Phe Tyr Gly Thr
95 100 105

Thr Tyr Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser
110 115 120

Ser
121

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 113 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
20 25 30

Ser Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45

Glu Trp Val Ser Val Ile Ser Gly Asp Gly Gly Ser Thr Tyr Tyr
50 55 60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
65 70 75

```
Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Gly Phe Asp Tyr Trp Gly Gln
                95                 100                 105

Gly Thr Leu Val Thr Val Ser Ser
               110         113
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 184 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Lys Gly Asn Val Asp Leu Val Phe Leu Phe Asp Gly Ser Met Ser
 1               5                  10                  15

Leu Gln Pro Asp Glu Phe Gln Lys Ile Leu Asp Phe Met Lys Asp
            20                  25                  30

Val Met Lys Lys Leu Ser Asn Thr Ser Tyr Gln Phe Ala Ala Val
            35                  40                  45

Gln Phe Ser Thr Ser Tyr Lys Thr Glu Phe Asp Phe Ser Asp Tyr
        50                  55                  60

Val Lys Arg Lys Asp Pro Asp Ala Leu Leu Lys His Val Lys His
            65                  70                  75

Met Leu Leu Leu Thr Asn Thr Phe Gly Ala Ile Asn Tyr Val Ala
            80                  85                  90

Thr Glu Val Phe Arg Glu Glu Leu Gly Ala Arg Pro Asp Ala Thr
            95                 100                 105

Lys Val Leu Ile Ile Ile Thr Asp Gly Glu Ala Thr Asp Ser Gly
           110                 115                 120

Asn Ile Asp Ala Ala Lys Asp Ile Ile Arg Tyr Ile Ile Gly Ile
       125                 130                 135

Gly Lys His Phe Gln Thr Lys Glu Ser Gln Glu Thr Leu His Lys
           140                 145                 150

Phe Ala Ser Lys Pro Ala Ser Glu Phe Val Lys Ile Leu Asp Thr
           155                 160                 165

Phe Glu Lys Leu Lys Asp Leu Phe Thr Glu Leu Gln Lys Lys Ile
           170                 175                 180

Tyr Val Ile Glu
          184
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 184 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Lys Gly Asn Val Asp Leu Ile Phe Leu Phe Asp Gly Ser Met Ser
 1               5                  10                  15

Leu Gln Pro Asp Glu Phe Gln Lys Ile Leu Asp Phe Met Lys Asp
            20                  25                  30

Val Met Lys Lys Leu Ser Asn Thr Ser Tyr Gln Phe Ala Ala Val
            35                  40                  45

Gln Phe Ser Thr Ser Tyr Lys Thr Glu Phe Asp Phe Ser Asp Tyr
            50                  55                  60

Val Lys Gln Lys Asp Pro Asp Ala Leu Leu Glu His Val Lys His
        65                  70                  75

Met Leu Leu Leu Thr Asn Thr Phe Gly Ala Ile Asn Tyr Val Ala
            80                  85                  90

Thr Glu Val Phe Arg Glu Glu Leu Gly Ala Arg Pro Asp Ala Thr
            95                 100                 105

Lys Val Leu Ile Ile Ile Thr Asp Gly Glu Ala Thr Asp Ser Gly
           110                 115                 120

Asn Ile Asp Ala Ala Lys Asp Ile Ile Arg Tyr Ile Ile Gly Ile
           125                 130                 135

Gly Lys His Phe Gln Thr Lys Glu Ser Gln Glu Thr Leu His Lys
       140                 145                 150

Phe Ala Ser Lys Pro Ala Ser Glu Phe Val Lys Ile Leu Asp Thr
           155                 160                 165

Phe Glu Lys Leu Lys Asp Leu Phe Thr Glu Leu Gln Lys Lys Ile
           170                 175                 180

Tyr Ala Ile Glu
       184
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Lys His Val Lys His Met Leu
 1               5       7
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Gly Tyr Ser Phe Thr Gly His Trp Met Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Ile His Pro Ser Asp Ser Glu Thr Arg Tyr Asn Gln Lys Phe
1 5 10 15

Lys Asp
17

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Gly Ile Tyr Phe Tyr Gly Thr Thr Tyr Phe Asp Tyr
1 5 10 12

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Arg Ala Ser Lys Thr Ile Ser Lys Tyr Leu Ala
1 5 10 11

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Ser Gly Ser Thr Leu Gln Ser
1 5 7

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Gln Gln His Asn Glu Tyr Pro Leu Thr
1 5 9

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Pro Lys Asn Ser Ser Met Ile Ser Asn Thr Pro
1 5 10 11

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

His Gln Ser Leu Gly Thr Gln
1 5 7

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

His Gln Asn Leu Ser Asp Gly Lys
1 5 8

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

His Gln Asn Ile Ser Asp Gly Lys
1 5 8

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Val Ile Ser Ser His Leu Gly Gln
1 5 8

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CACTTTGGAT ACCGCGTCCT GCAGGT 26

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CATCCTGCAG GTCTGCCTTC AGGTCA 26

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Met Ile Ala Pro Ala Ser Ser Ser Thr Arg Tyr Asn Gln Lys Phe
1 5 10 15

Lys Asp

17

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 121 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Thr
20 25 30

Gly His Trp Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45

Glu Trp Val Gly Met Ile Ala Pro Ala Ser Ser Ser Thr Arg Tyr
50 55 60

Asn Gln Lys Phe Lys Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
65 70 75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ala Arg Gly Ile Tyr Phe Tyr Gly Thr
95 100 105

Thr Tyr Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser
110 115 120

Ser
121

WHAT IS CLAIMED IS:

1. A humanized anti-CD11a antibody which binds specifically to human CD11a I-domain.

2. The humanized anti-CD11a antibody of claim 1 which binds to epitope MHM24 on CD11a.

3. A humanized anti-CD11a antibody which binds human CD11a with a K_d value of no more than about 1×10^{-8} M.

4. A humanized anti-CDIIa antibody which has an IC50(nM) value of no more than about 1nM for preventing adhesion of Jurkat cells to normal human epidermal keratinocytes expressing ICAM-1.

5. A humanized anti-CDIIa antibody which has an IC50 (nM) value of no more than about 1nM in the mixed lymphocyte response assay.

6. The humanized anti-CD11a antibody of claim 1 having a heavy chain variable region comprising the amino acid sequence of CDR1 (SEQ ID NO:10), CDR2 (SEQ ID NO:11) and CDR3 (SEQ ID NO:12) of humanized antibody MHM24 (F(ab)-8.

7. The humanized anti-CD11a antibody of claim 6 comprising the amino acid sequence of SEQ ID NO:5.

8. The humanized anti-CD11a antibody of claim 1 having a light chain variable region comprising the amino acid sequence of CDR1 (SEQ ID NO:13), CDR2 (SEQ ID NO:14) and CDR3 (SEQ ID NO:15) of humanized MHM24 F(ab)-8.

9. The humanized anti-CD11a antibody of claim 8 comprising the amino acid sequence of SEQ ID NO:2.

10. The humanized anti-CD11a antibody of claim 1 having a light chain variable region comprising the amino acid sequence of SEQ ID NO:5 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:2.

11. The humanized anti-CD11a antibody of claim 1 which is a full length antibody.

12. The humanized anti-CD11a antibody of claim 11 which is a human IgG.

13. The antibody of claim 1 which is an antibody fragment.

14. The antibody fragment of claim 13 which is a $F(ab')_2$.

15. The antibody of claim 1 which is labeled with a detectable label.

16. The antibody of claim 1 which is immobilized on a solid phase.

17. The antibody of claim 1 which is conjugated to a cytotoxic agent.

18. A method for determining the presence of CD11a protein comprising exposing a sample suspected of containing the CD11a protein to the antibody of claim 1 and determining binding of said antibody to the sample.

19. A kit comprising the antibody of claim 1 and instructions for using the antibody to detect the CD11a protein.

20. Isolated nucleic acid encoding the antibody of claim 1.

21. A vector comprising the nucleic acid of claim 20.

22. A host cell comprising the vector of claim 21.

23. A process of producing a humanized anti-CD11a antibody comprising culturing the host cell of claim 22 so that the nucleic acid is expressed.

24. The process of claim 23 further comprising recovering the humanized anti-CD11a antibody from the host cell culture.

25. The process of claim 24 wherein the humanized anti-CD11a antibody is recovered from the host cell culture medium.

```
          1       10        20        30        40
MHM24     DVQITQSPSYLAASPGETISINC[RASKTISKYLA]WYQEKPGKTNKLLIY
           • •     • •  • •••• •                •    ••
F(ab)-8   DIQMTQSPSSLSASVGDRVTITC[RASKTISKYLA]WYQQKPGKAPKLLIY
                                    ••• •
humκI     DIQMTQSPSSLSASVGDRVTITC[RASQSISNYLA]WYQQKPGKAPKLLIY

                    60        70        80        90
MHM24     [SGSTLQS]GIPSRFSGSGSGTDFTLTISSLEPEDFAMYYC[QQHNEYPLT]
                   •                    •    •
F(ab)-8   [SGSTLQS]GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC[QQHNEYPLT]
           •• • •                                   • •• •
humκI     [AASSLES]GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC[QQYNSLPWT]

          100
MHM24     FGTGTKLELKR
            •   • •
F(ab)-8   FGQGTKVEIKR
humκI     FGQGTKVEIKR
```

FIG._1A

```
          1       10        20        30        40
MHM24     EVQLQQPGAELMRPGASVKLSCKASGYSFT[GHWMN]WVRQRPGQGLEWIG
           ••• •• ••  • ••   •                 •  •    •
F(ab)-8   EVQLVESGGGLVQPGGSLRLSCAASGYSFT[GHWMN]WVRQAPGKGLEWVG
                                    •• • ••• •          •
humIII    EVQLVESGGGLVQPGGSLRLSCAASGFTFS[SYAMS]WVRQAPGKGLEWVS
RhIgG1    EVQLVESGGGLVQPGGSLRLSCAASGYSFT[GHWMN]WVRQAPGKGLEWVG

          50 a      60        70        80 abc    90
MHM24     [MIHPSDSETRLNQKFKD]KATLTVDKSSSSAYMQLSSPTSEDSAVYYCAR
                   •          •• ••    •••• • •• •••  •
F(ab)-8   [MIHPSDSETRYNQKFKD]RFTISVDKSKNTLYLQMNSLRAEDTAVYYCAR
           • •••••• • •••• •       • •
humIII    [VISGDGGSTYYADSVKG]RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR
RhIgG1    [MIAPASSSTRYNQKFKD]RFTISVDKSKNTLYLQMNSLRAEDTAVYYCAR

                  100       110
MHM24     [GIYFYGTTYFDY]WGQGTTLTVSS
F(ab)-8   [GIYFYGTTYFDY]WGQGTLVTVSS
            ••••••••
humIII    [G--------FDY]WGQGTLVTVSS
RhIgG1    [GIYFYGTTYFDY]WGQGTLVTVSS
```

FIG._1B

```
                130        140        150        160        170
                 •          •          •          •          •
humCD11a   KGNVDLVFLFDGSMSLQPDEFQKILDFMKDVMKKLSNTSYQFAAVQF
rhCD11a          I
                 β1                    α1                   β2

                180        190        200        210
                 •          •          •          •
humCD11a   STSYKTEFDESDYVKRKDPDALLKHVKHMLLLTNTFGAINYVATEV
rhCD11a                    Q          E
                β2'        α2         α3                  α4

                      230        240        250        260
                       •          •          •          •
humCD11a   FREELGARPDATKVLIIITDGEATDSGNIDAAKDIIRYIIGIGKHFQ
rhCD11a
                            β3                  α5        β4

                270        280        290        300        310
                 •          •          •          •          •
humCD11a   TKESQETLHKFASKPASEFVKILDTFEKLKDLFTELQKKIYVIE
rhCD11a                                                    A
                α6                   β5         α7
```

FIG._2

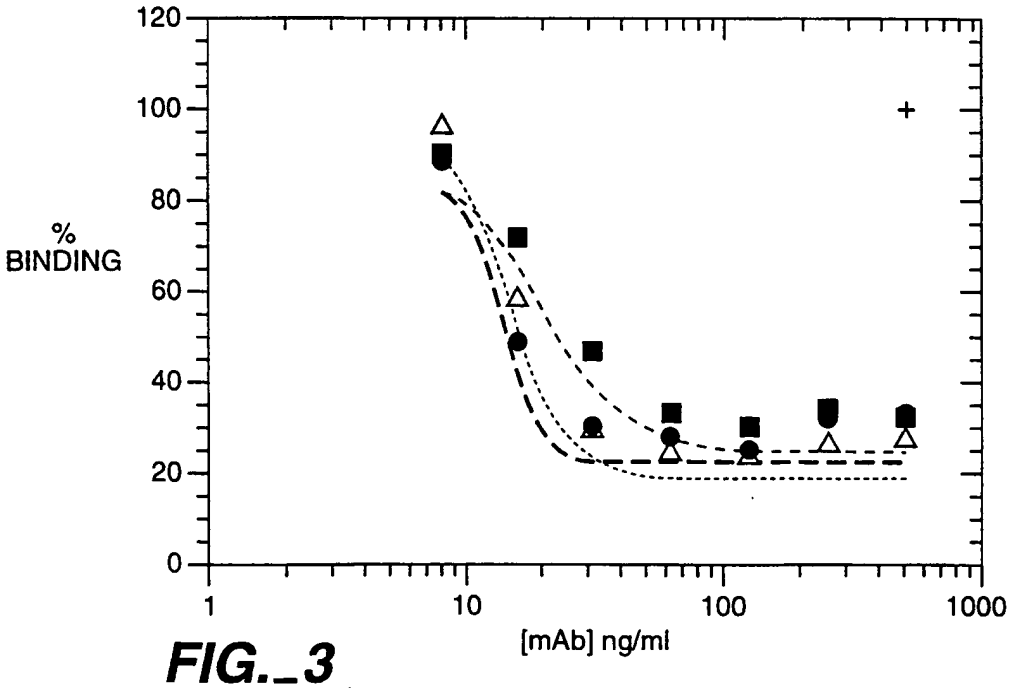


FIG._3

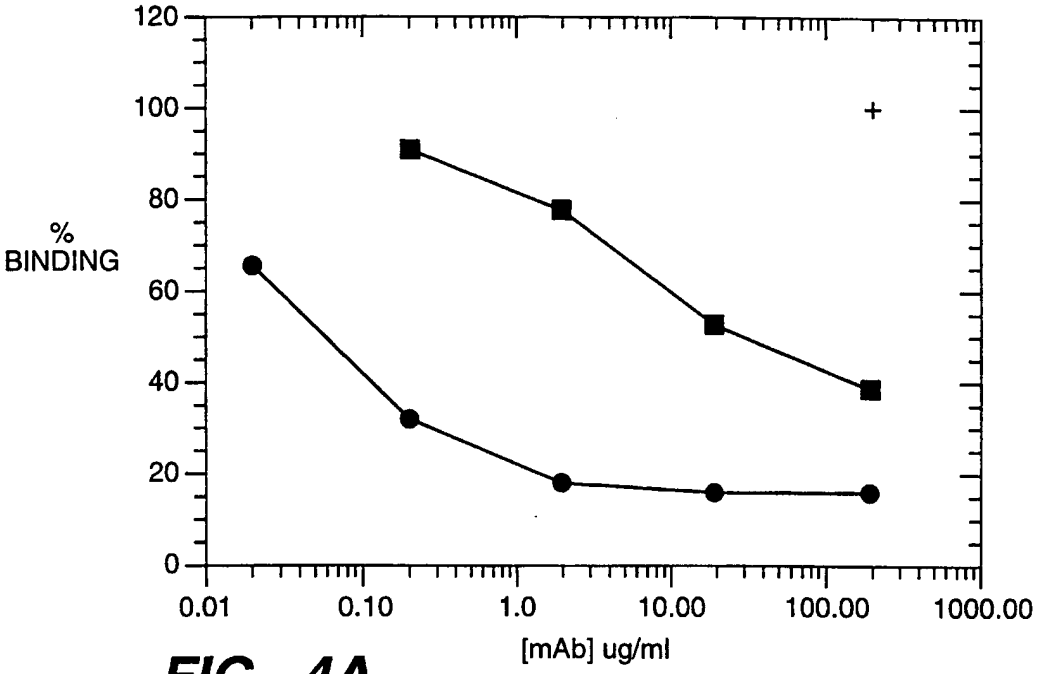

120
100
80
60
40
20
0
%
BINDING
0.01
0.10
1.0
10.00
100.00
1000.00
[mAb] ug/ml

FIG._4A

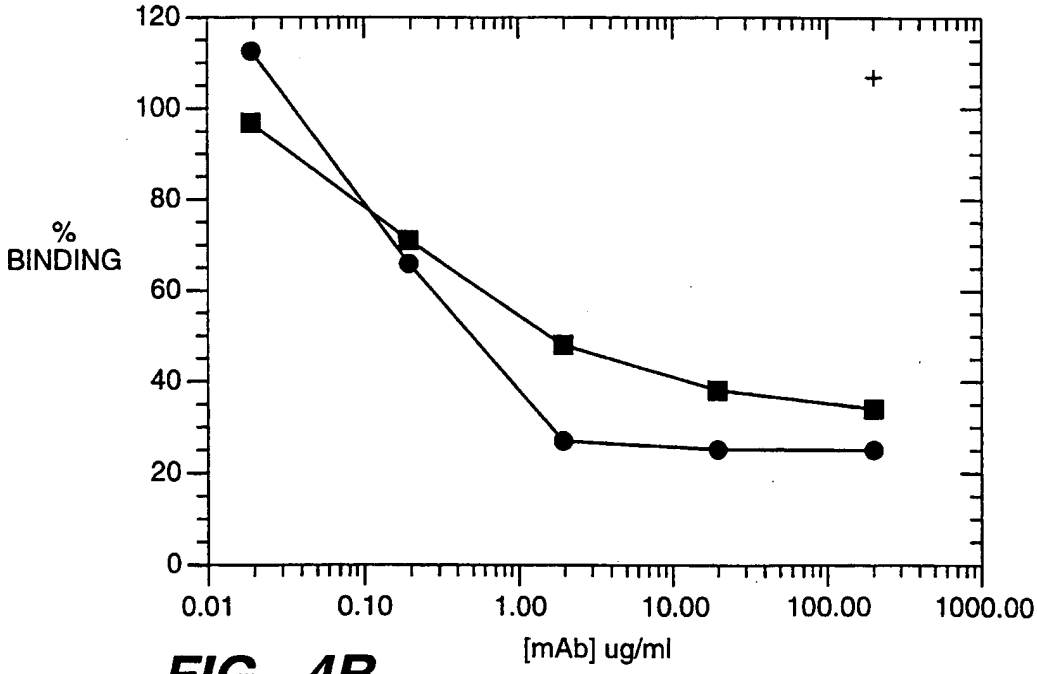

120
100
80
60
40
20
0
%
BINDING
0.01
0.10
1.00
10.00
100.00
1000.00
[mAb] ug/ml

FIG._4B

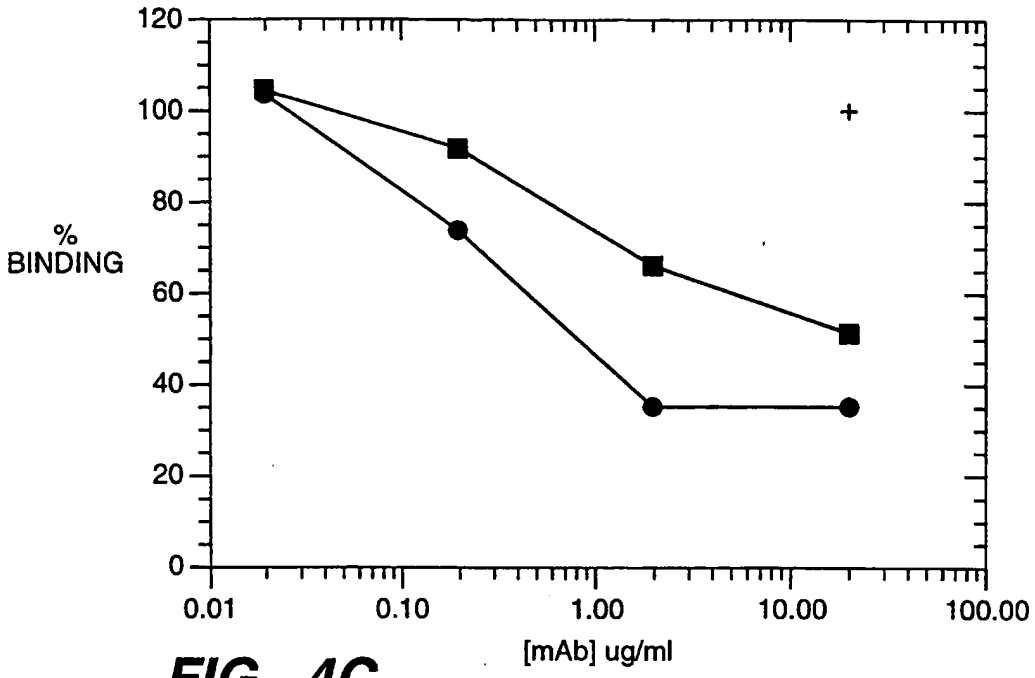

120
100
80
60
40
20
0
%
BINDING
0.01
0.10
1.00
10.00
100.00
[mAb] ug/ml

FIG._4C

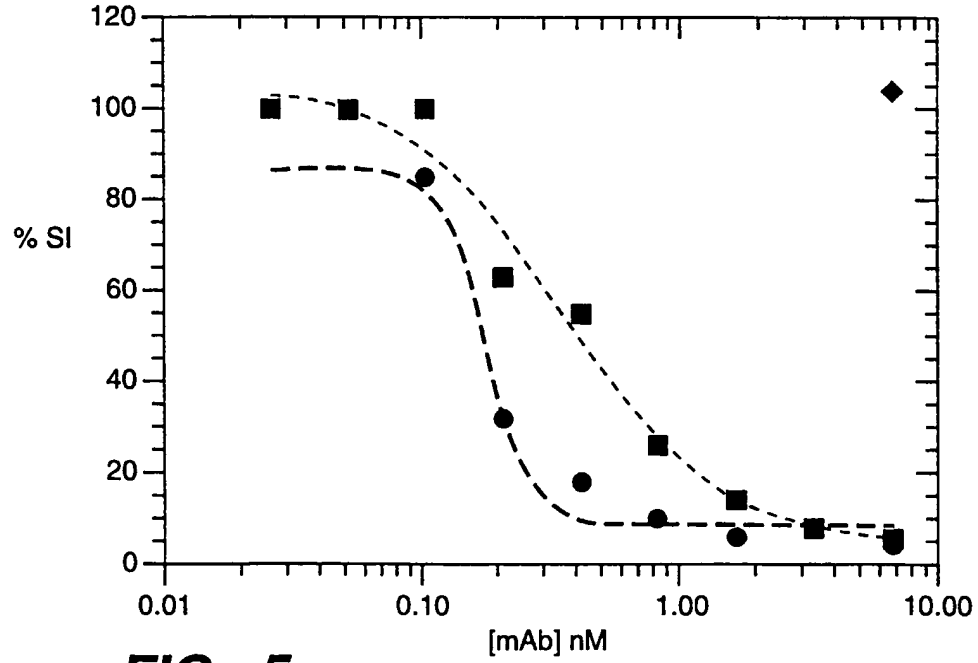

120
100
80
60
40
20
0
% SI
0.01
0.10
1.00
10.00
[mAb] nM

FIG._5

INTERNATIONAL SEARCH REPORT

Interr ıal Application No
PCT/US 97/19041

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/62 C07K16/28 C07K16/46

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. HILDRETH ET AL.: "A human lymphocyte-associated antigen involved in cell-mediated lympholysis." EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 13, no. 3, March 1983, WEINHEIM, GERMANY, pages 202-208, XP002054055 cited in the application see the whole document --- -/--	1-25

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publicationdate of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of theinternational search	Date of mailing of the international search report
30 January 1998	20/02/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nooij, F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter ıal Application No
PCT/US 97/19041

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category ˚	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	C. EDWARDS ET AL.: "Identification of amino acids in the CD11a I-domain important for binding of the leukocyte function-associated antigen (LFA-1) to intercellular adhesion molecule-1 (ICAM-1)." THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 21, 26 May 1995, BALTIMORE, MD, USA, pages 12635-12640, XP002054056 see abstract see figure 1 ---	1-25
A	WO 96 32478 A (GENENTECH, INC.) 17 October 1996 see claims ---	1-25
A	EP 0 440 351 A (MERCK & CO. INC.) 7 August 1991 see claims ---	1-25
A	P. CARTER ET AL.: "Humanization of an anti-p185HER2 antibody for human cancer therapy." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 89, no. 10, May 1992, WASHINGTON, DC, USA, pages 4285-4289, XP000275844 cited in the application see the whole document ---	1-25
P,X	W. WERTHER ET AL.: "Humanization of an anti-lymphocyte function-associated antigen (LFA-1) monoclonal antibody and reengineering of the humanized antibody for binding to rhesus LFA-1." THE JOURNAL OF IMMUNOLOGY, vol. 157, no. 11, 1 December 1996, BALTIMORE, MD, USA, pages 4986-4995, XP002054057 see the whole document -----	1-25

INTERNATIONAL SEARCH REPORT

...ormation on patent family members

Inter... nal Application No
PCT/US 97/19041

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9632478 A	17-10-96	AU 5435996 A	30-10-96
EP 440351 A	07-08-91	AU 635996 B	08-04-93
		AU 6984391 A	25-07-91
		CA 2034574 A	20-07-91
		CA 2034593 A	20-07-91
		EP 0438312 A	24-07-91
		JP 6054697 A	01-03-94
		JP 6054698 A	01-03-94